The Interplay Between Adipose Tissue and Renal Cell Carcinoma: Decoding the Obesity Paradox

Miguel Mendes Ferreira

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Biologia Celular e Molecular

2019

The Interplay Between Adipose Tissue and Renal Cell Carcinoma: Decoding the Obesity Paradox

Miguel Mendes Ferreira
MSc in Cell and Molecular Biology
Department of Biology
Faculty of Sciences, University of Porto (FCUP), Portugal
Rua do Campo Alegre 1021/1055, 4169-007 Porto
up201303022@fc.up.pt
2019

Supervisor:
Ricardo Jorge Teixeira Ribeiro M.D., PhD
Researcher, Tumour & Microenvironment Interactions Group, Biomedical Engineering Institute (INEB), Institute for Research and Innovation in Health, University of Porto (i3S)
Affiliated Invited Professor, Laboratory of Genetics and Environmental Health Institute, Faculty of Medicine, University of Lisbon
Medical Doctor, Department of Clinical Pathology, Centro Hospitalar e Universitário de COIMBRA

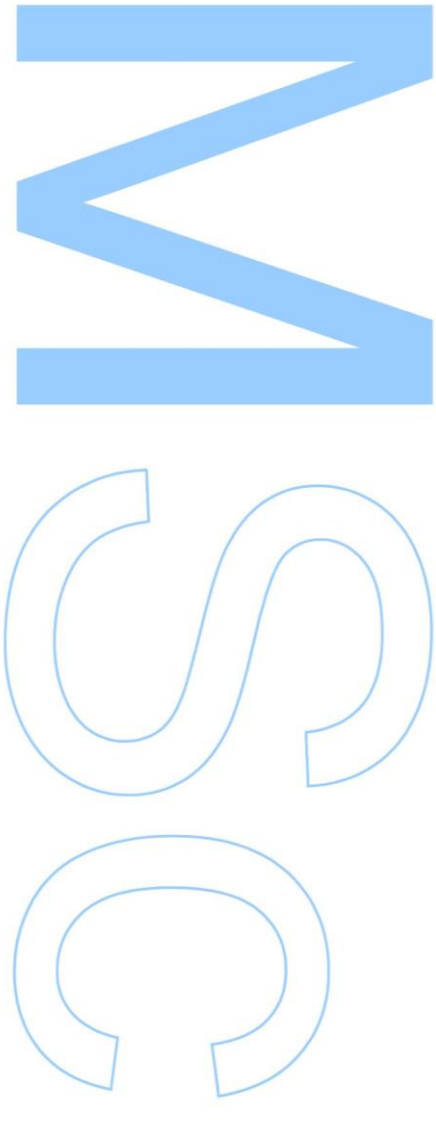
Co-supervisor:
Alexandra Maria Monteiro Gouveia, PhD
Researcher, Ageing and Stress Group, Institute for Molecular and Cell Biology (IBMC), Institute for Research and Innovation in Health, University of Porto (i3S)
Invited Assistant Professor, Faculty of Medicine and Faculty of Nutrition and Food Sciences, University of Porto

INSTITUTO DE INVESTIGAÇÃO E INOVAÇÃO EM SAÚDE
UNIVERSIDADE DO PORTO

U.PORTO

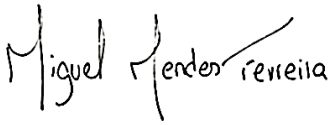
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,
Porto, _____/_____/_____



Authorship declaration

I, Miguel Mendes Ferreira, student number 201303022 from the Master's Degree in Cell and Molecular Biology, Faculdade de Ciências da Universidade do Porto (2016/2019), hereby declare that I am the sole author of this Dissertation Project and that the contents herein reflect my personal work. I certify and declare on my honor the authenticity of this work. In such wise, I further declare I understand the consequences of the violation of these conditions.



Miguel Mendes Ferreira

Porto, November 15, 2019

"Jim Watson and I have probably made a most important discovery. We have built a model for the structure of de-oxy-ribose-nucleic-acid (read it carefully) called D.N.A. for short (…) Our structure is very beautiful. D.N.A. can be thought of roughly as a very long chain with flat bits sticking out. The flat bits are called the "bases" (…) It is like a code. If you are given one set of letters you can write down the others. Now we believe that the D.N.A. is a code. That is, the order of the bases (the letters) makes one gene different from another gene (…) You can now see how Nature makes copies of the genes. (…) In other words we think we have found the basic copying mechanism by which life comes from life. The beauty of our model is that the shape of it is such that only these pairs can go together, though they could pair up in other ways if they were floating about freely (…) Read this carefully so that you understand it."

Francis Crick's Letter to Son (1953)

Acknowledgments

Antes de mais, gostava de referir que o meu crescimento pessoal e científico foi além do que era expectável para mim quando iniciei este projeto. Desde já quero deixar o meu profundo agradecimento ao Professor Ricardo Ribeiro, que me orientou, não só cientificamente, mas também como pessoa. Agradeço-lhe pela oportunidade que me deu de desenvolver um projeto e a liberdade que me proporcionou para tomar decisões foram imprescindíveis para que crescesse como investigador. De seguida, gostaria de agradecer à Professora Alexandra Gouveia pela co-orientação e disponibilidade em ajudar-me com problemas técnicos que enfrentamos todos os dias.

Não menos importante, gostaria de demonstrar o meu enorme obrigado à Professora Maria José Oliveira que, de uma forma ou de outra, ainda que nem sempre com imenso tempo, tenha disponibilizado uma enorme quantidade de tempo comigo. Espero que não tenha sido um desperdício. Obrigado por me ter levado a puxar por mim próprio. Por me ter feito crescer. Por me ter feito acreditar em mim. Neste momento, é completamente indiscútivel agradecer ao grupo que me acolheu. Vocês fizeram a caminhada um bocadinho mais fácil e extremamente divertida. À Ângela, à Patrícia e à Tânia por me terem respondido sempre às minhas perguntas “parvas” e por estarem sempre disponíveis. Por me fazerem rir todos os dias. À Ângela Magalhães, à Maria José Silveira e à Flávia Pereira por me terem ajudado na prática e teórica do que é ser um investigador. Às Beatrizes por, independentemente de serem elementos novos, terem contribuido para novas aprendizagens (inclusive astrologia e coisas). Gostaria de dar um particular agradecimento à Andreia por ser a parceira da gordura nesta caminhada e por toda a ajuda disponibilizada. À Ana Borges, Ana Luísa e Sara, obrigado por me fazerem sentir melhor todos os dias. Se pudesse voltar atrás e escolher outro grupo, não o faria, vocês tornaram-se imprescindíveis para mim. Por último, gostaria de dar uma obrigado particular à Flávia Castro, que, sabendo ou não, é uma das pessoas mais brilhantes que já conheci. Obrigado por todo o sacrifício que tiveste de fazer para que esta tese saísse como está. Pela força que me dás. Obrigado. Obrigado por serem o grupo que eu não merecia mas recebi. Obrigado por serem o melhor grupo de sempre!

De seguida gostaria de agradecer aos meus amigos de longa data (Narciso, Nuno, Steve, Diogo, Preto, Zé Eduardo). Obrigado pela motivação que me deram para a concretização deste projeto. Obrigado pelos momentos de boa disposição que passamos a discutir ciência. À Ana Sofia por, por muito tempo que passemos separados encontrar-me sempre na altura certa. Por perguntar como estou e como está a correr, mesmo que a distância seja muita. À Teresa, por me ter acompanhado nos últimos três anos e ter sido a

força que eu precisava para conseguir tudo o que consegui até agora. Obrigado. Por último, à Fi. Foste sempre a âncora na minha vida. Desde que te conheci que caminhamos juntos. Obrigado por me teres aturado, em todas as fases da minha vida, boas e más. Obrigado!

Por último, mas certamente o mais importante, gostaria de agradecer aos meus pais e ao meu irmão. Por fazerem mais do que o esforço financeiro, por me terem educado a ser como sou, por fazerem de mim alguém com princípios e moral. Espero que percebam que mais do que meu, isto é vosso. É resultado de todo um esforço que não é só meu. É vosso. Não há palavras suficientes que possam descrever a forma como os agradecimentos vos deverão ser feitos. Obrigado.

Obrigado a todos que de uma forma ou outra passaram pela minha vida e contribuiram para o meu percurso. De um modo ou do outro foram todos importantes para mim. Espero um dia poder retribuir.

Obrigado

Abstract

Renal cell carcinoma (RCC) denotes a common group of chemoresistant cancers derived from the renal epithelium. RCC accounts for over 90% of all the cancers in the kidney and includes over 10 histological and molecular subtypes. Clear cell renal cell carcinoma (ccRCC) is the most common RCC subtype and accounts for most cancer-related deaths. Importantly, complete surgical resection remains the only curative treatment for RCC, for it is of critical importance to develop new therapeutic, less invasive strategies as curative intents for this disease.

Compelling evidence exists over the influence of obesity on cancer development. Excess adipose tissue (AT) accrual is a well-established factor for cancer incidence. Notably, RCC is among the cancers for which this effect is more evident. However, RCC obese patients at diagnosis experience longer survival than non-obese patients, a phenomenon termed “obesity paradox”. Notwithstanding, the influence of the adipose depot in RCC, and particularly, in ccRCC is yet largely unexplored.

This project focused on providing a better understanding of the adipose tissue/ccRCC interface and unveil potential mechanisms through which adipose tissue exerts its influence on ccRCC. Following on this premise, the work herein developed aims to contribute to the current state of knowledge over the bidirectional crosstalk between adipose tissue and cancer by: i) exploring the intrinsic differences between adjacent tumour and distant adipose depots; ii) addressing the influence of AT secreted factors on hypoxia, inflammatory-, epithelial to mesenchymal transition- (EMT) and stem-related gene expression in ccRCC cell lines; and iii) evidencing the role of adipose depot-derived soluble factors on established hallmarks of cancer, namely, proliferation and apoptosis of 786-O and Caki-1 cells. The resulting data suggest that, in fact, both perirenal (PrAT) and subcutaneous (ScAT) adipose depots exert an influence over clear cell renal cell carcinoma primary tumours, by increasing the expression of genes that contribute to progression and development of ccRCC and decreasing tumour viability. However, these counterintuitive data could not be observed in metastatic ccRCC Caki-1 cell line.

In conclusion, this work provides a better understanding of the influence of site-specific adipose depots for ccRCC and, importantly, conveys promising data to educate future research and expand the knowledge over the AT/cancer interface, and ultimately, giving prospective future approaches for the development of novel therapeutic targets to impact not only ccRCC itself, but the surrounding microenvironment, in particular, the adipose depot.

Keywords: obesity, adipose tissue, clear cell renal cell carcinoma, tumour microenvironment.

Resumo

O carcinoma de células renais (CCR) engloba um grupo de neoplasias quimioresistentes derivadas do epitélio renal. Mais de 90% dos cancros renais em adultos são CCR, que pode ser subdividido em 10 subtipos histológicos e moleculares. Destes, o CCR de células claras (CCRcc) é o mais comum e com maior mortalidade associada. Presentemente, a resecção cirúrgica completa é a única abordagem terapêutica disponível para pacientes com este cancro, relevando-se a importância do desenvolvimento de novas terapias e estratégias cirúrgicas menos invasivas para o seu tratamento.

Atualmente é aceite que a obesidade influencia o desenvolvimento de cancro. A acumulação excessiva de tecido adiposo (TA) está estabelecida como sendo um fator relevante na incidência desta doença, sendo o CCR um dos tipos em que este efeito é mais evidente. No entanto, em pacientes obesos diagnosticados com CCR observa-se uma taxa de sobrevivência superior comparativamente a pacientes não obesos, instituindo-se o "paradoxo da obesidade". Contudo, a influência dos depósitos de TA sobre o CCR, e sobre o CCRcc em particular, é uma área de estudo pouco desenvolvida.

O presente projeto focou-se na análise da interface TA/CCRcc e investigação dos potenciais mecanismos pelos quais o TA exerce influência sobre o CCRcc. Seguindo estas premissas, o trabalho desenvolvido no âmbito deste projeto teve como objetivo contribuir para o estado atual do conhecimento sobre a interação bidirecional do TA e o cancro através: i) do estudo das diferenças intrínsecas entre depósitos de TA adjacentes/distantes de tumores; ii) da análise da influência de fatores secretados pelo TA na expressão de genes em linhas celulares de CCRcc, genes esses relacionados com hipoxia, inflamação, transição epitelial-mesenquimal (TEM) e estaminalidade; e iii) do estudo do papel de fatores solúveis derivados de depósitos de TA sobre características do cancro bem estabelecidas, nomeadamente, sobre a proliferação e apoptose de células 786-O e Caki-1. Os resultados reunidos nesta tese sugerem que tanto o TA perirrenal (TAPr) como o TA subcutâneo (TASc) exercem influência sobre tumores primários de CCRcc, através, por um lado, do aumento da expressão de genes que contribuem para o aumento da progressão e desenvolvimento do CCRcc e, por outro, da diminuição da viabilidade tumoral. No entanto, estas observações contraditórias não foram encontradas em Caki-1, uma linha celular metastática de CCRcc.

Em suma, o trabalho desenvolvido permite uma melhor compreensão da influência de depósitos de TA encontrados em locais específicos sobre o CCRcc. Adicionalmente, os dados reunidos neste projeto acrescentam ao estado atual do conhecimento, podendo sustentar linhas de investigações posteriores focadas na interface TA/cancro. Ultimamente,

os resultados do trabalho aqui descrito podem incitar abordagens inovadoras para o desenvolvimento de terapêuticas não só focadas no CCRcc em si, mas também no microambiente tumoral e no TA em particular.

Palavras-chave: obesidade, tecido adiposo, carcinoma de células renais de células claras, microambiente tumoral.

List of contents

List of contents 7
List of figures 9
List of tables 10
List of abbreviations 11
INTRODUCTION 16
RENAL CELL CARCINOMA - A METABOLIC DISEASE 16
Overview 16
Mechanistic Insights - Genetic Basis and Pathways 18
Tumour Microenvironment 22
ADIPOSE TISSUE 25
Functional Characterization and Adipose Identity 25
Developmental Signature and Transcriptional Regulation 28
Adipose Tissue Microenvironment and Cellular Crosstalk 29
Adipose Secretory Profile and Metabolism 32
ADIPOSE TISSUE AND CANCER INTERFACE 33
Epidemiologic Evidence for Obesity-Related Cancer 33
Adipose Tissue Microenvironment and Cancer Progression 34
Tumour-Adipocyte Specific Interactions 38
ADIPOSE TISSUE AT THE NEXUS OF RENAL CELL CARCINOMA 41
AIMS 42
MATERIALS AND METHODS 43
Clinical Samples and Adipose Tissue *Ex Vivo* Culture 43
Cell Lines Maintenance and Culture Conditions 43
Adipocyte Size Measurements 44
Cell Proliferation 44
Apoptosis Assay 45
Zymography 45
RNA Extraction and Purification 46
Complementary DNA First-Strand Synthesis 47
Quantitative Real-Time PCR 47
Statistical Analysis 48
RESULTS 49
ADIPOSE TISSUE CHARACTERIZATION 49
Perirenal and Subcutaneous Adipose Tissue Have Distinct Morphological Features 49

Perirenal Adipose Tissue Displays a Trend to Increase Thermogenic-Selective Genes.....51
Perirenal Adipose Depot Secretes Higher Amounts of Matrix Metalloproteinases.....53
ADIPOSE TISSUE AND CLEAR CELL RENAL CELL CARCINOMA CROSSTALK 54
Adipose Tissue Alters the Gene Expression Profile of 786-O Cells.....54
Adipose Tissue Soluble Factors Have No Effect on Caki-1 Cells Gene Expression.....56
Adipose Tissue-Derived CM Inhibits Cell Proliferation of 786-O Cells.....58
Adipose Tissue Derived CM Promotes 786-O Apoptosis.....60
DISCUSSION 62
CONCLUDING REMARKS AND FUTURE PERSPECTIVES 67
REFERENCES.....69

List of figures

Figure 1 | Estimated global renal cell carcinoma cancer incidence.. 16
Figure 2 | Renal cell carcinoma staging. 17
Figure 3 | *VHL* loss-of-function in renal cell carcinoma....18
Figure 4 | Renal cell carcinoma is a disease of 3p loss 20
Figure 5 | Pathways beyond VHL-HIF 21
Figure 6 | Tumour microenvironment....22
Figure 7 | Adipocytes have distinct phenotypic features....25
Figure 8 | UCP1-dependent thermogenesis 27
Figure 9 | Brown and white adipogenesis 28
Figure 10 | Adipose tissue promotes malignant transformation and tumour development.34
Figure 11 | Obese tumour microenvironment 35
Figure 12 | Cancer cells and cancer-associated adipocytes crosstalk....39
Figure 13 | Perirenal and subcutaneous adipocytes have distinct sizes....50
Figure 14 | Gene expression profile of perirenal (PrAT) and subcutaneous (ScAT) adipose depots 52
Figure 15 | MMP2 and MMP9 proteolytic activity in perirenal- and subcutaneous-derived conditioned media....53
Figure 16 | Adipose tissue-derived conditioned media induces alterations in mRNA relative expression of ccRCC- (A), EMT- (B) and stem- (C) related genes in 786-O cells. 55
Figure 17 | Adipose tissue-derived conditioned media does not appear to impact mRNA relative expression of tumour promoting- (A), EMT- (B) and stem- (C) related genes in Caki-1 cells....57
Figure 18 | Proliferation rates of 786-O and Caki-1 cell lines....59
Figure 19 | Effect of perirenal and subcutaneous adipose tissue-derived conditioned media on the cell apoptosis of 786-O (A) and Caki-1 cells (B)....60

List of tables

Table 1 | Clear cell renal cell carcinoma lines. 44
Table 2 | Gene assays for qRT-PCR 48

List of abbreviations

3p	Short arm of chromosome 3
AdipoR	Adiponectin receptor
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANGPTL4	Angiopoietin-like protein 4
APC	Adenomatous polyposis coli protein
ASC	Adipose-derived stem cells
AT	Adipose tissue
ATM	Adipose tissue macrophage
ATP	Adenosine triphosphate
BAP1	BRCA1 associated protein 1
BARD1	BRCA1-associated RING domain protein 1
BAT	Brown adipose tissue
BCL2	B-cell lymphoma 2 gene
BMI	Body mass index
BMP	Bone morphogenetic protein
BRCA1	Breast cancer type 1 susceptibility gene
C/EBPα	CCAAT/enhancer binding protein α
CAA	Cancer-associated adipocyte
CCL	Chemokine ligand of the CC family
CCND1	Cyclin D1
ccRCC	Clear cell renal cell carcinoma
CD	Cluster of differentiation
CDH1	Cadherin 1
CM	Conditioned medium
c-MET	Tyrosine-protein kinase Met
c-MYC	MYC proto-oncogene

COL6α3	Collagen type VI alpha 3 chain
CT	Computed tomography
CTRL	Control
CXCL	Chemokine ligand of the CXC family
DKK	Dickkopf-related protein
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EBF2	Early B cell factor 2
ECM	Extracellular matrix
EdU	5-ethynyl-2´-deoxyuridine
EHMT1	Euchromatic histone-lysine N-methyltransferase 1
EIF4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal–regulated kinases
EVA1	Epithelial V-like antigen 1
FA	Fatty acid
FABP4	Fatty acid-binding protein 4
FASN	Fatty acid synthase gene
FBN2	Fibrillin 2
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	FGF receptor
GSH	Glutathione (reduced)
H&E	Haematoxylin and eosin
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HRE	HIF response element
IL	Interleukin
ILC	Innate lymphoid cell
IMS	Intermembrane space

iNKT	Invariant natural killer cell
iNOS	Inducible nitric oxide synthase
LCFA	Long-chain fatty acid
LOH	Loss of heterozygosity
MCP1	Monocyte chemoattractant protein 1
MDSC	Myeloid-derived suppressor cells
MET	Hepatocyte growth factor receptor
MMP	Matrix metalloproteinase
Mrna	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
mTOR	Mechanistic target of rapamycin kinase
mTORC	Mechanistic target of rapamycin complex
MYF5	Myogenic factor 5
NAD	Nicotinamide adenine dinucleotide
NBCS	New-born calf serum
NGF	Nerve growth factor
NRG4	Neuregulin 4
OBR	Leptin receptor
OCT4	Octamer-binding transcription factor 4
OD	Optical density
PAI1	Plasminogen activator inhibitor 1
PAX7	Paired box protein 7
PBRM1	Polybromo-1 protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDGF	Platelet-derived growth factor
PDGFRβ	Platelet-derived growth factor receptor beta
PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
PPARγ	Peroxisome proliferator-activated receptor gamma

PrAT	Perirenal adipose tissue
PRDM16	PR domain-containing 16
PTEN	Phosphatase and tensin homologue
qRT-PCR	Quantitative real-time PCR
RCC	Renal cell carcinoma
RHEB	Ras homolog enriched in brain
RNA	Ribonucleic acid
ROS	Radical oxygen species
RPMI	Roswell Park Memorial Institute
S6K1	Ribosomal protein S6 kinase
ScAT	Subcutaneous adipose tissue
SD	Standard deviation
SETD2	SET domain containing protein 2
SFRP	Secreted frizzled-related protein
SLC	Solute carrier family
SLUG	SNAI2
SNAI	Snail family transcriptional repressor
SNAIL	SNAI1
STAT3	Signal transducer and activator of transcription 3
SWI/SNF	Switch/sucrose non-fermenting complex
TAM	Tumour-associated macrophage
TBX1	T-box transcription factor 1
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor
TF	Transcription factor
TGFβ	Transforming growth factor beta
TME	Tumour microenvironment
TMEM26	Transmembrane protein 26
TNF	Tumour necrosis factor
T_{reg}	Regulatory T cell
TSC	Tuberous sclerosis complex
TWIST	Twist family bHLH transcription factor

UCP1	Uncoupled protein 1
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VHL	von Hippel-Lindau tumour suppressor gene
WAT	White adipose tissue
WIF1	Wnt inhibitory factor 1
ZEB	Zinc finger E-box binding homeobox
ZFP	Zinc-finger protein
αSMA	Alfa smooth muscle actin

INTRODUCTION

RENAL CELL CARCINOMA - A METABOLIC DISEASE

Overview

Renal cell carcinoma (RCC) is the 13th cause of cancer-related death and one of the ten most diagnosed cancers worldwide, accounting for over 300 000 new cases and 140 000 deaths every year (**Figure 1**)[1,2].

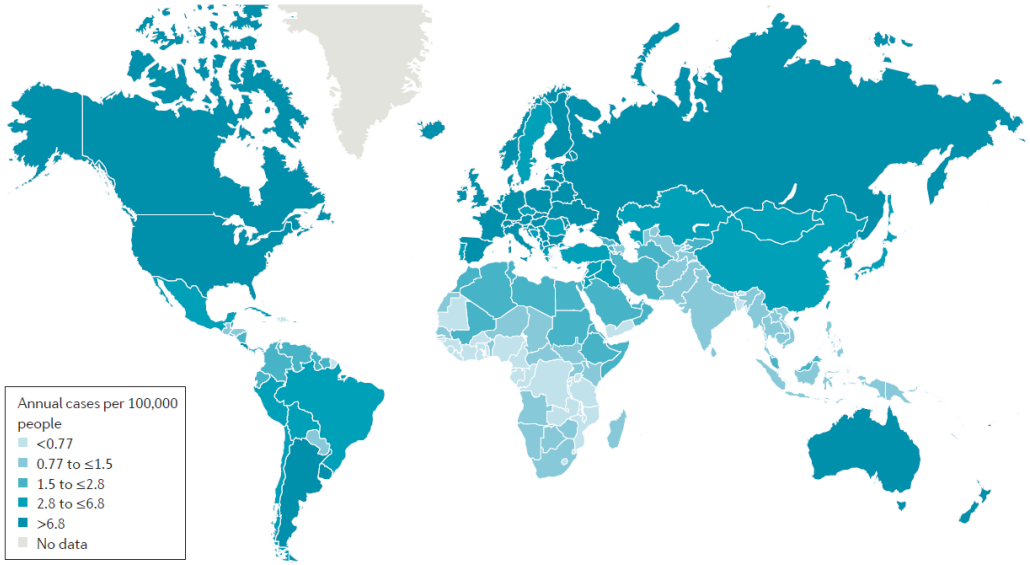


Figure 1 | **Estimated global renal cell carcinoma cancer incidence.** From Hsieh et al. *Nat. Rev. Dis. Primers* 2017[3].

Nearly one third of all RCC patients have disseminated metastasis at presentation and about half of the patients eventually succumb to the disease[4]. Importantly, RCC denotes not a single disease but a common group of chemoresistant cancers that originate from the renal parenchyma and include a spectrum of distinguishable histopathological subtypes with distinct molecular profiles, clinical outcomes and treatment responses. Clear cell renal cell carcinoma (ccRCC) is the most common and aggressive subtype of RCC and is characterized by the presence of glycogen- and lipid-laden cells, implicating several alterations in ccRCC development, including fatty acid and glucose metabolic dysfunctions[3,5-7].

Recent exome sequencing studies have highlighted the increased intra-tumour[8,9] and inter-tumour[5] heterogeneity found in ccRCC patients, leading to an intricate complex of clinical outcomes. Even though relative survival rates have been improved in the past few years, a global increase of RCC incidence has been reported. In general, the use of non-invasive radiological techniques has contributed to this finding. Furthermore, increments in global excess body weight, hypertension and cigarette smoking are critical to further increase RCC prevalence[2]. Staging of RCC reflects local invasiveness, lymph node involvement and metastases assessment and is mainly accomplished with CT analysis (**Figure 2**)[10].

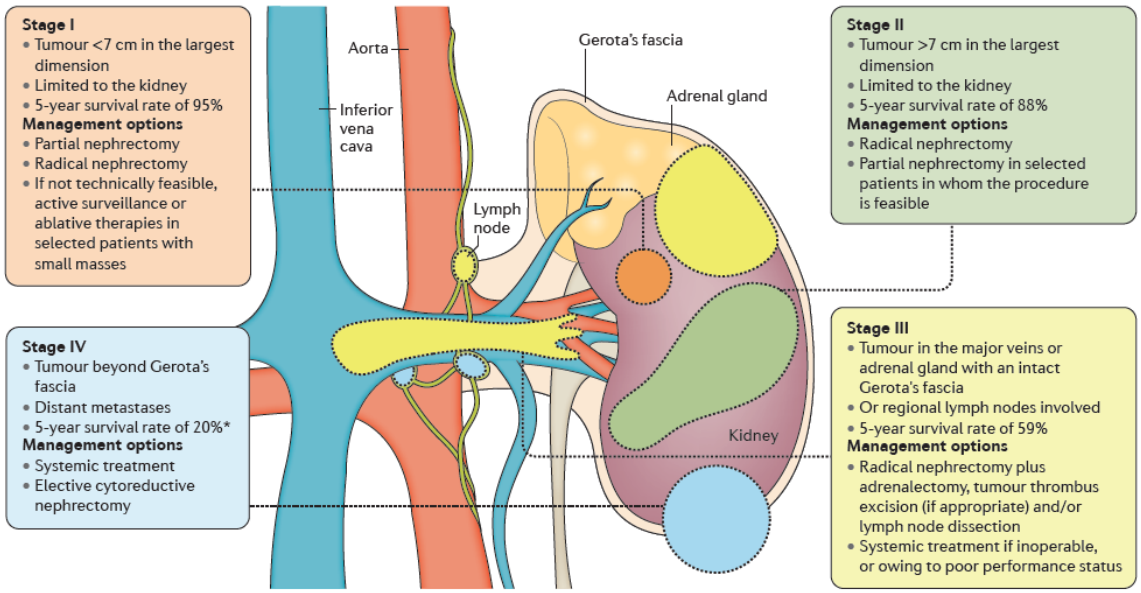


Figure 2 | **Renal cell carcinoma staging**. Tumour staging is mainly accomplished through computed tomography and reflects local invasiveness, lymph node involvement and metastases. Stages I and II are mainly confined to the kidney and surgical procedures remain the main curative treatment for these patients. Stage III reflects tumours enclosed within Gerota's fascia limits. In turn, stage IV RCC reflects distant metastases and treatment options mainly focus on systemic treatments, including Sunitinib, Sorafenib, Bevacizumab and Everolimus. From Hsieh et al. *Nat. Rev. Dis. Primers* 2017[3].

For patients with surgically resectable ccRCC, the standard procedure remains partial or radical nephrectomy with curative intent. However, approximately 30% of patients with ccRCC will eventually develop metastasis, which require systemic treatment with targeted agents and/or immune checkpoint inhibitors. Complete surgical resection remains, however, the only curative treatment for RCC[3,11].

Mechanistic Insights - Genetic Basis and Pathways

Dysregulated metabolism is a hallmark of cancer. Tumours reprogram metabolic statuses to obtain sufficient energy and biosynthetic building blocks to sustain malignant cell proliferation[12]. Genetically, loss of the short arm of chromosome 3 (3p) is a central feature in ccRCC occurring in over 90% of patients. The von Hippel-Lindau tumour suppressor gene (*VHL*) is located on chromosome 3p25 and its biallelic loss through 3p loss and concurrent *VHL* somatic mutation, promoter hypermethylation or deletion is considered the defining primary event in this type of cancer[5,6,13]. Recent whole genome sequencing studies highlighted this as the key driver event in ccRCC tumorigenesis[14]. The VHL protein is part of an E3 ubiquitin ligase complex which recognizes, binds and targets hypoxia inducible factors (HIFs), in an oxygen-dependent manner, for proteasomal degradation. *VHL* loss-of-function leads to an aberrant accumulation of HIF proteins, that become constitutively stabilized in the early carcinogenic process, and direct transcriptional programs that promote cellular proliferation, angiogenesis and modulate intracellular metabolic processes (**Figure 3**)[15,16].

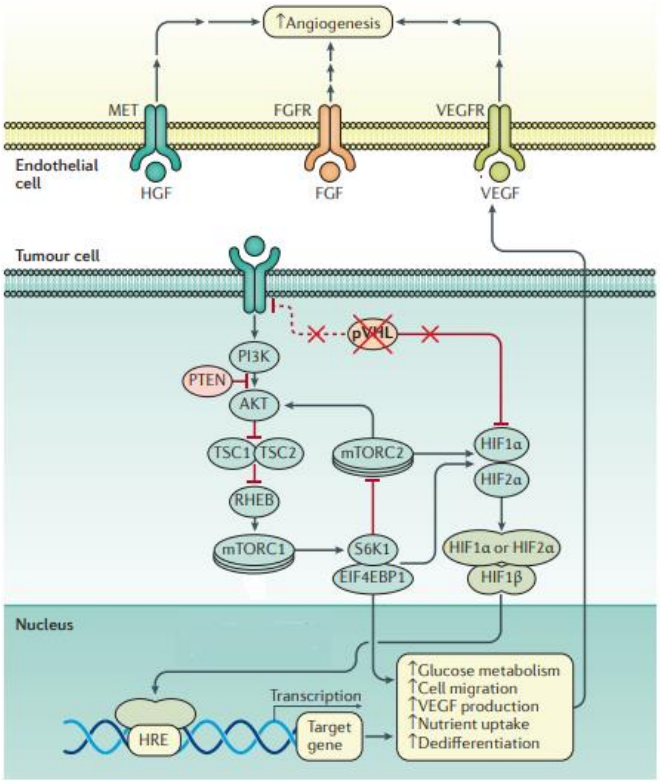


Figure 3 | ***VHL* loss-of-function in renal cell carcinoma.** *VHL* inactivation is the most frequent genetic manifestation in clear cell renal cell carcinoma. In the absence of VHL, HIF transcriptional targets expression increases leading to changes in cellular metabolism. HIF transcriptional targets include VEGF, FGF and HGF increasing angiogenesis and cell growth. HGF signalling through MET and tumour promoting mutations enhance PI3K/AKT/mTOR activity. Both VHL-HIF and PI3K/AKT/mTOR signalling pathways are intrinsically associated with metabolic regulation and constitute the underlying evidence that RCC is essentially a metabolic disease. Adapted from Hsieh et al. *Nat. Rev. Dis. Primers* 2017[3].

Loss of Chromosome 3p

Large-scale cancer genomic sequencing projects have identified recurrent mutations in tumour suppressor genes other than *VHL* involved in chromatin and/or histone modifications, including *PBRM1*, *BAP1* and *SETD2*, making clear the role of the altered epigenetic control of gene expression to the ccRCC carcinogenic processes[17].

The complex interplay between genetics and epigenetics in RCC may be illustrated by the role *PBRM1* has in ccRCC pathogenesis. *PBRM1* is part of the SWI/SNF chromatin-remodelling complex. In ccRCC, this gene functions as a tumour suppressor gene, and its inactivation is associated with dysregulated DNA repair, proliferation and differentiation[18]. Even though the mechanisms by which *PBRM1* mutations contribute to RCC remain elusive, the regulation of genes that encode proteins essential to proliferation (*e.g.* p21) and cell-adhesion/cell-signalling (*e.g.* E-cadherin) are thought to play a role in the pro-oncogenic effect that the ablation of this gene has in ccRCC carcinogenesis[19]. In addition, loss of this chromatin remodelling factor rescues deficient VHL-mediated DNA damage and replication stress, contributing to cellular fitness and promoting proliferation[20]. In concordance, *PBRM1* has been implicated on the limitation of HIF and STAT3 transcription outputs to over-amplify upon *VHL* loss, suggesting *PBRM1* inactivation as the second oncogenic driver in ccRCC[21].

BAP1 encodes a nuclear deubiquitinase, which is inactivated in 15% of ccRCC patients, and forms a multiprotein complex with BRCA1 and BARD1, regulating multiple cellular pathways including DNA damage, cell cycle and cell proliferation processes[22,23]. Acquisition of *BAP1* mutations have been associated with high tumour grades and mTORC1 activation, further implicating BAP1 in onco-promoting processes, including EMT, growth sustainment and survival[22,24]. Recently, BAP1 was associated to increase ferroptotic cell death-resistance in RCC cell lines de-repressing SLC7A11, a transporter of extracellular cystine, enabling the continuous uptake of cystine and GSH synthesis, promoting tumour development[25].

SETD2 encodes a histone methyltransferase that is associated with open heterochromatin and decreased CpG methylation. Therefore, *SETD2* mutations alter the expression and functional structure of a myriad of genes by directly repressing gene expression or influencing the spliceosome machinery and impacting splice-variants expression. *SETD2* loss-of-function impairs an efficient gene transcription and has been associated with defects on DNA break repair mechanisms and increased genomic instability, ultimately, driving malignant cell transformation[19,26,27].

Notably, four of the most commonly mutated genes in RCC (*VHL*, *PBRM1*, *BAP1* and *SETD2*) are located in chromosome 3p (**Figure 4**). Allelic loss of 3p is a nearly universal event in RCC, and therefore, results in haploinsufficiency for four of the tumour suppressor genes are usually related to RCC aggressiveness[5,28].

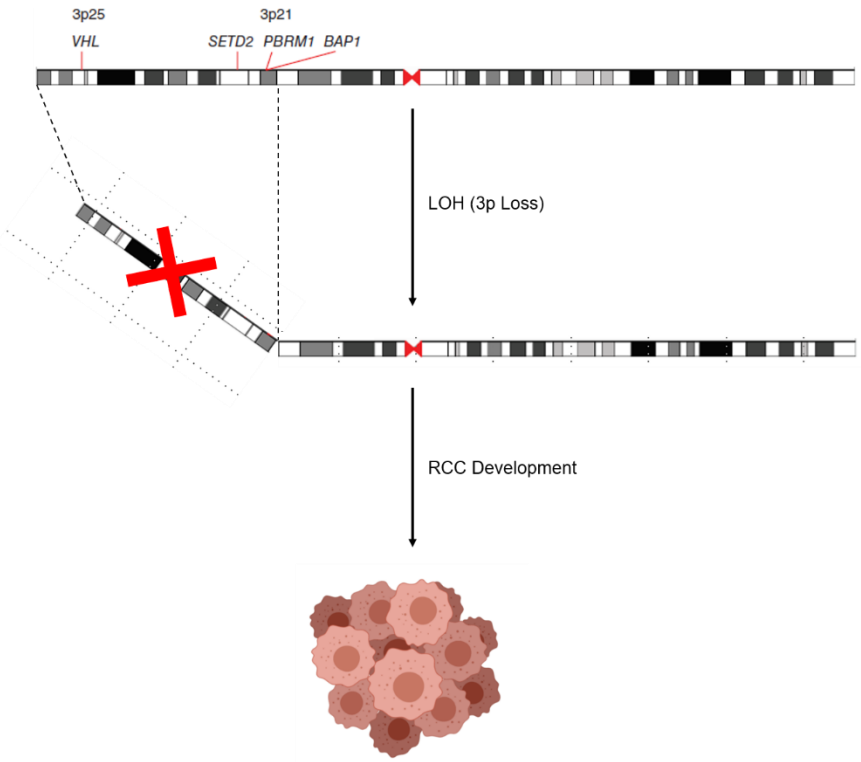


Figure 4 | **Renal cell carcinoma is a disease of 3p loss**. Four of the most commonly mutated genes in RCC are located within the short arm of chromosome 3p. 3p loss is a nearly universal event in the development of RCC. Loss of *VHL*, PBRM1, *SETD2* and *BAP1* through 3p loss is a common event in RCC. Adapted from Hakimi et al. *Nat. Genet.* 2013[28].

Related Signalling Pathways

Recurrently tumour promoting mutations found in ccRCC also include genes in PI3K-AKT-mTOR signalling and p53-related pathways, leading to a series of dysregulated cell functions, including apoptosis, cell survival, angiogenesis, cell-cycle regulation, among others (**Figure 3**)[3,5,6]. In addition, frequently epigenetic mechanisms in ccRCC contribute for mTOR increased activity that may lead to increased expression of *c-MET* and *BCL2* transcripts as well as the inhibition of *PTEN*.

Clear cell renal cell carcinoma is further aggravated by altered epigenetic regulation on WNT-β-catenin and TGFβ signalling pathways (**Figure 5**).

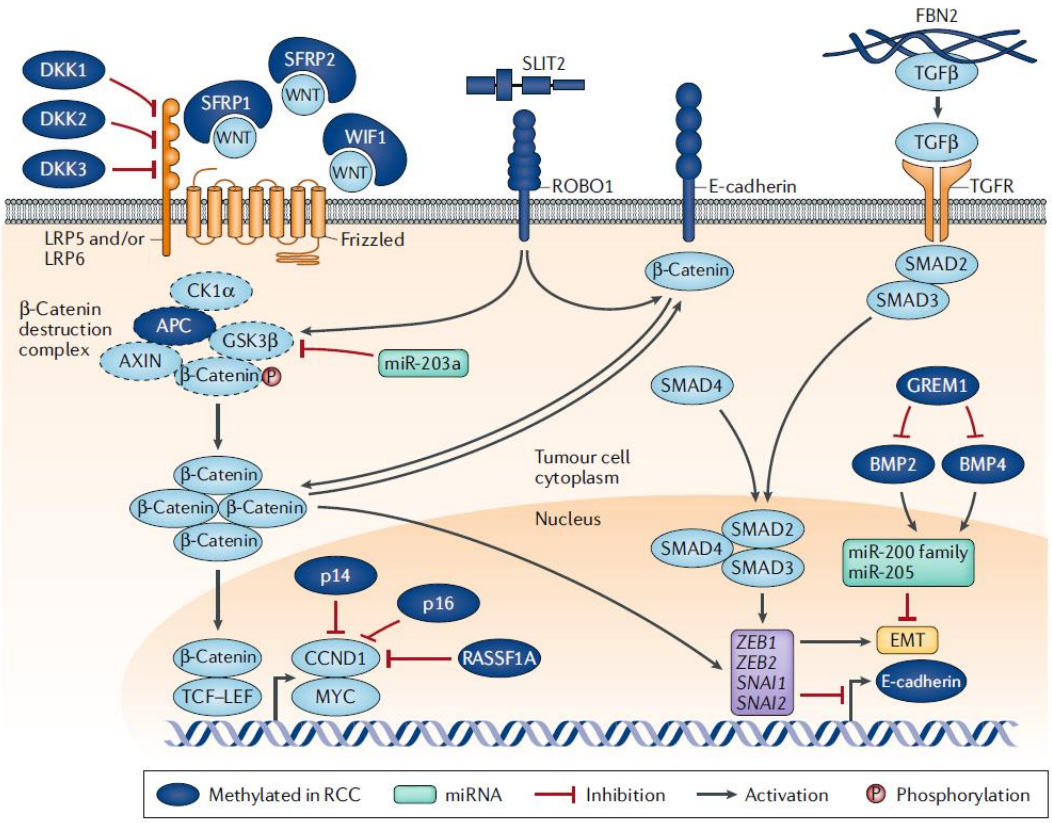


Figure 5 | **Pathways beyond VHL-HIF**. WNT signalling is increased through epigenetic deregulation of WNT antagonists. These include DKK1, DKK2 and DKK3, which bind to WNT receptors, impairing signalling; and SFRP1,SFRP2 and WIF1 which directly bind WNT, preventing the bind of WNT to its receptors. In addition, promoter methylation of APC, a component of the β-catenin destruction complex, enables β-catenin accumulation and, consecutively, upregulation of its ocogenic target genes. Frequent mutations in FBN2 increase TGFβ signalling, further increasing the transcription of tumour promoting genes, including epithelial to mesenchymal transcription factors. From Joosten et al. *Nat. Rev. Urol.* 2018[17].

In the absence of WNT binding to its receptor complex, β-catenin is phosphorylated and targeted for degradation. However, binding of WNT to its receptor complex leads to accumulation and translocation of β-catenin to the nucleus where it associates with DNA-binding transcription factors of the TCF/LEF family, promoting transcription of target genes such as cyclin D1 (*CCND1*) and *c-MYC*. In RCC, promoter methylation of the genes encoding WNT antagonists and APC result in loss of negative regulation of WNT signalling and lead to the accumulation of β-catenin and its downstream targets. Additionally, in *VHL*-deficient ccRCC patients, β-catenin can be targeted for degradation. However, this process is often impaired owing to promoter methylation and consequent inactivation of WNT pathway inhibitors. Notably, β-catenin has also an extremely important role in maintaining cell-cell adhesion by binding to the cytoplasmic domain of E-cadherin, and thus preventing its proteasomal degradation, contributing to the maintenance of an epithelial phenotype. Promoter methylation of *CDH1*, which encodes E-cadherin, or reduced expression through a HIF1α-dependent manner in RCC results in β-catenin availability and ultimately, to

increased transcription of its target genes, which include EMT transcription factors (*e.g.* *TWIST*, *SNAI1*, *SNAI2*, and *ZEB1)*, which further repress E-cadherin expression in RCC. Importantly, TGFβ signalling triggers the activation of SMAD proteins that directly stimulate the expression of the above mentioned EMT transcription factors. In addition, TGFβ signalling further enhances mTOR activity, contributing to ccRCC pathogenesis, via PI3K and AKT activation. In RCC, promoter methylation of *FBN2*, a microfibrillar component of the ECM that sequesters TGFβ, leads to release of TGFβ from the ECM and consequent downstream signalling, further contributing to RCC progression.

Tumour Microenvironment

The tumour microenvironment (TME) is composed of an intricate milieu of non-transformed cells and acellular components that include immune cells, fibroblasts, stromal, epithelial, endothelial, fat and cancer stem cells, as well as the surrounding ECM constituents and the inflammatory mediators that these cells secrete. Elements of the TME can interact with tumour cells directly or through paracrine factors that contribute to the development, progression and therapy resistance in RCC (**Figure 6**)[12,17,29,30].

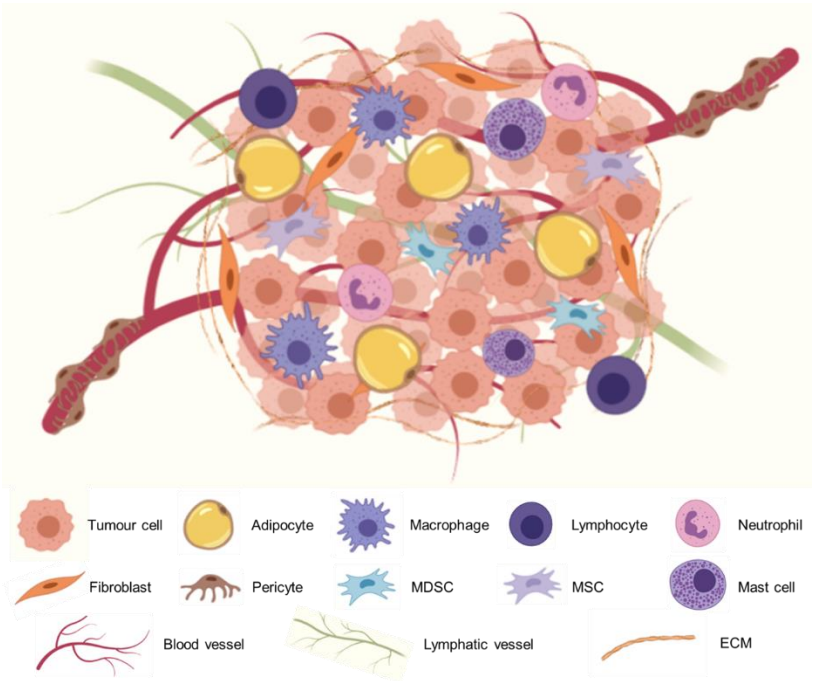


Figure 6 | **Tumour microenvironment**. Tumour cells are surrounded by a myriad of cellular and non-cellular components that constitute its microenvironment. These components include endothelial cells of the vascular and lymphatic systems, fibroblasts, a complex of immune cells, myeloyd-derived supressor cells (MDSC) mesenchymal stem cells (MSC), adipocytes and preadipocytes, and extracellular matrix (ECM) components. Cancer cells are capable of modulating its environment in order to initiate metastases and/or promote its growth.

Immune Landscape

Tumour cells are able to develop immunosuppressive mechanisms that ultimately enable tumour progression[31]. Immune cells infiltrate the TME and create a niche that is capable of modulating nearly every aspect of tumour development. $CD4^+$ and $CD8^+$ T cells target antigenic tumour cells to prevent cancer cell growth[32]. However, tumour cells often evade host anti-tumour response by reducing its immunogenicity and activating immunosuppressive and/or immunoevasive pathways, decreasing gene transcription of genes that encode tumour-associated antigens, HLAs and constituents of the antigen processing and presentation machinery, which render cells invisible to $CD8^+$ cytotoxic T lymphocytes. Furthermore, upregulation of immunosuppressive molecules, such as programmed cell death 1 ligand 1 (PDL1), IL-6, IL-10, TGFβ and VEGF, render cancer cells immune resistance[17,33]. These factors recruit immunosuppressive myeloid-derived suppressor cells (MDSCs) to the tumour which, in turn, further represses antitumour responses and recruits and/or promote differentiation of cells in tumour-associated macrophages (TAMs), regulatory T (T_{regs}) cells, as well as other immunosuppressive cells[17]. In fact, short-lived patients with ccRCC have extremely high levels of T helper 2 cells and T_{regs} infiltration[34,35].

Metabolic Landscape

Alterations in cellular metabolism has now been widely considered as a hallmark of cancer and provide a route for cancer progression and development. Clear cell renal cell carcinoma cells are glycogen- and lipid-laden, and therefore, possess dysregulated fatty acid and glucose metabolic processes that ultimately, may be representative of ccRCC aggressiveness[7]. *VHL* inactivation is a central event in ccRCC, either caused by gene deletion, mutation and/or silencing via promoter methylation, therefore leading to HIF accumulation and consequent HIFα metabolic rearrangements, including glycolysis, fatty acid synthesis and glycogen synthesis modifications[36]. In fact, recurrent mutations in ccRCC are associated with genes that are related to the dysregulation of at least one metabolic pathway that is mediated by oxygen, iron, energy or nutrient sensing, further supporting the view of ccRCC as a metabolic disease[15]. A metabolic shift towards increased dependence on the pentose phosphate shunt, decreased activities of AMPK and the Krebs cycle, increased glutamine transport and higher fatty acid production has already been demonstrated to correlate with disease aggressiveness. This poor ccRCC prognosis resembles the Warburg metabolic phenotype[5,28,36]. Therefore, it is somehow expected that

obesity is closely associated with ccRCC development and progression, given that ccRCC is fundamentally a metabolic disease[15].

ADIPOSE TISSUE

Adipose tissue (AT) is increasingly being recognized as a crucial organ that contributes to human health processes, through both systemic and local-specific functions[37]. This organ has gained increased interest for its potential in regulating body energy expenditure, impacting obesity as a current global epidemic. Obesity stems from the imbalance between energy intake and expenditure. If energy consumption constantly exceeds energy expenditure, the surplus of energy is stored in adipocytes and adipose mass is expanded. This process is particularly important since obesity is associated with an intricate array of diseases including type 2 diabetes, cardiovascular diseases, insulin resistance and several types of cancer[38-40].

Functional Characterization and Adipose Identity

AT is a critical organ in regulating energy homeostasis composed mainly of adipocytes. However, several other cell types can be found within this tissue, including vascular endothelial cells, neurons, preadipocytes, fibroblasts and a myriad of immune cells. The crosstalk among these different cell types modulates adipose tissue activity on a depot and/or type-specific manner[41].

Mammalian adipose tissue can be broadly divided into two functionally distinct classes of fat, white (WAT) and brown (BAT), depending on whether this depot is mainly composed of white or brown adipocytes, respectively (**Figure 7**).

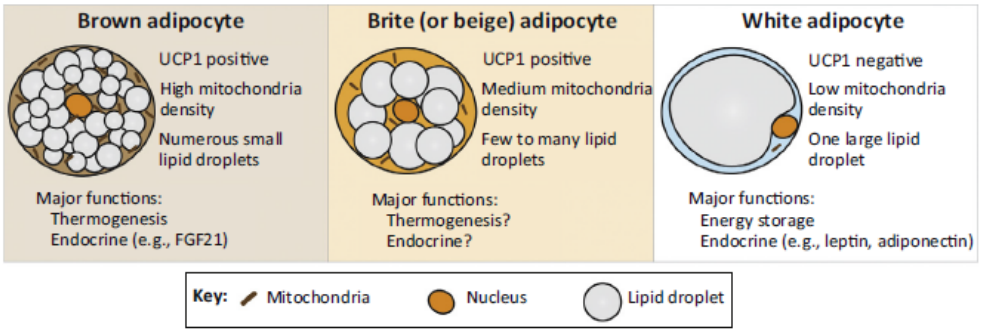


Figure 7 | **Adipocytes have distinct phenotypic features**. Adipocytes are broadly divided into three classes: brown, beige and white adipocytes that have distinct functional and morphological characteristics. Brown adipocytes are usually smaller than white and beige fat cells and possess numerous lipid droplets and numerous UCP1-expression mitochondria. These cells main function resorts in their adaptive thermogenic capacity that is highly dependent on UCP1. White adipocytes mainly function as an energy storage cell and possess a large unilocular lipid droplet that occupies nearly all the space within the cell. In turn, beige adipocytes are thought to emerge in white adipose depots through transdifferentiation or *de novo* differentiation from preadipocytes and upon stimulation (*e.g.* cold or β-adrenergic stimuli) present similar phenotypic traits to brown adipocytes. From Sanchez-Gurmaches et al. *Trends Cell Biol.* 2016[42].

Whereas WAT is mainly responsible for the storage and release of energy in response to systemic demands, BAT contributes to whole energy body expenditure via thermogenic processes, which has set the attention towards the modulation of the adipose depot to promote weight loss[43-45].

White Adipose Tissue

White adipocytes are the predominant type of adipocyte and contain large unilocular lipid droplets and a low mitochondrial content. These cells are important regulators of energy homeostasis and are responsible for the storage of fuel and release of a variety of adipokines, including leptin and adiponectin that regulate insulin sensitivity, lipid metabolic processes and satiety. WAT is distributed throughout the human body in subcutaneous and visceral depots and can both increase in size (hypertrophy) or number (hyperplasia), and its expansion is a physiological process critical to the protection of other organs from lipotoxicity[42,46]. However, increased adiposity might lead to an ectopic accumulation of triglycerides and underlying metabolic syndrome, which includes impaired glucose metabolism and insulin resistance[47]. Importantly, the overall distribution of WAT has profound effects on human health. Excess visceral fat is highly correlated with development of metabolic disease. However, increased subcutaneous mass has been associated with improved glucose tolerance[48].

Brown Adipose Tissue

Brown adipocytes are enriched in uncoupled protein 1 (UCP1) mitochondria and possess small multilocular lipid droplets, mainly contributing to thermogenic processes. These cells burn substrates, including glucose and fatty acids, consuming energy by dissipating heat through a process known as adaptive thermogenesis[49,50]. This process is highly dependent on the capability of UCP1 to uncouple oxidative phosphorylation from ATP production (**Figure 8**)[51,52]. In unstimulated BAT, UCP1 is mainly inactive due to the inhibitory action of purine nucleotides. However, upon cold exposure or adrenergic stimuli, UCP1 expression is increased, initiating the thermogenic process[50,53,54]. Even though adult humans were thought to not possess thermogenic fat cells, fluorodeoxyglucose positron emission tomography scans demonstrated metabolically active fat tissue in humans, as given by glucose incorporation rates[55-57]. In humans, BAT is mainly located within the supraclavicular, paravertebral and periadrenal areas, and is a highly vascularized and densely innervated tissue in order to enable the dissipation of heat throughout the body and

to sense adrenergic stimuli upon cold exposure, respectively. This constitutes an intricate complex that regulates mammal thermogenesis and enables body temperature maintenance[44,50,58].

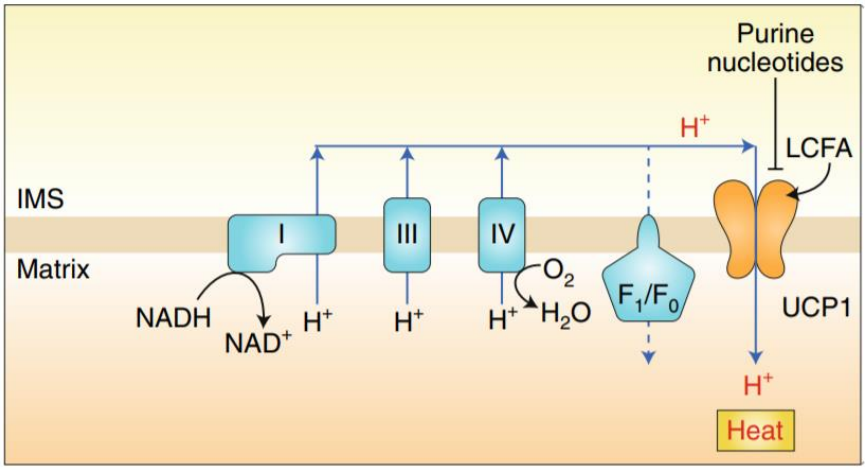


Figure 8 | **UCP1-dependent thermogenesis.** The thermogenic ability of brown and beige adipocytes is conferred by uncoupling protein 1 (UCP1). UCP1 is located in the inner mitochondrial membrane and uncouples oxidative phosphorylation from ATP synthesis, releasing energy by dissipating the mitochondrial proton gradient at the expense of ATP production. This inducible proton leak can be repressed through the action of purine nucleotides and promoted through the action of long chain fatty acids. Adapted from Chouchani et al. *Nat. Metab.* 2019[59].

Beige Adipocytes - Emerging Thermogenic Fat Cells

Interestingly, emerging evidence has unveiled the role of a second class of thermogenic fat cells, termed beige or brite adipocytes. Clusters of thermogenic adipocytes can be found within some WAT depots, in response to prolonged cold exposure or β3-adrenergic stimuli. Beige adipocytes share characteristics of both white and brown fat, depending on environmental cues[60]. When unstimulated, beige fat cells appear similar to white adipocytes. However, upon stimulation, beige cells acquire a multilocular appearance with densely packed UCP1-expressing mitochondria, which confers them thermogenic characteristics. This process is referred to as the 'browning' of WAT[43]. Recent reports have highlighted the role of thermogenic fat cells in humans. However, previously considered BAT in humans has now been revisited and described as beige fat depots, consistent with the view that brown adipocytes are lost during adulthood and beige cells develop as an evolutive conserved mechanism to provide plasticity in adaptive thermogenesis[61].

White, brown and beige adipocytes are distinct types of cells that greatly differ in their function. Whether WAT hypertrophic growth underlies the development of metabolic syndrome, BAT and/or beige fat cells activity may revert this process and improve metabolism. In an attempt to improve specific selection of different fat cells, several marker genes have already been described to distinguish white, brown and beige adipocytes. These markers include SLC7A10 for white fat; TBX1, TMEM26 and CD137 for beige adipocytes; and UCP-1, EVA1 and PRDM16 for brown fat cells[45,60-63].

Developmental Signature and Transcriptional Regulation

Fat pads in adult humans seem to have a cohort of adipocyte progenitor cells in the vascular stromal fraction of adipose depots that ensure adipocyte renewal upon environmental stimuli. Adipogenesis refers to the process whereby fibroblast-like precursor cells commit to the adipogenic lineage, giving rise to mature adipocytes in a two-step process. Initially, a multipotent mesenchymal progenitor commits to the adipogenic lineage, followed by differentiation, where preadipocytes undergo growth arrest, accumulate lipids and form functional adipocytes (**Figure 9**)[42,64,65].

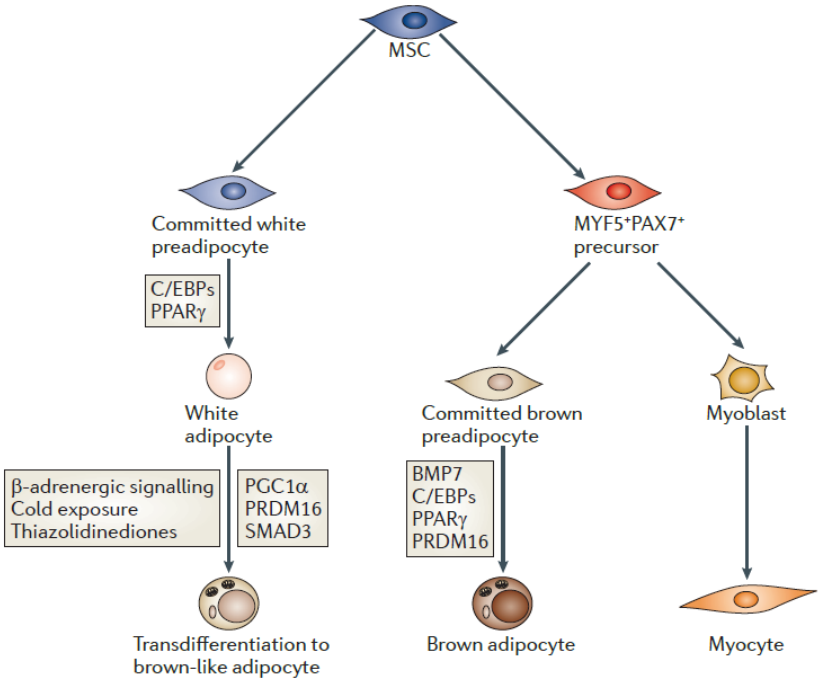


Figure 9 | **Brown and white adipogenesis**. White and brown adipocytes stem from different precursor cells. Brown adipocytes share a common $MYF5^{+}PAX7^{+}$ ancestor with myocytes. This precursor cell drives brown adipogenesis upon the influence of bone morphogenetic proteins (BMPs) and PR domain-containing 16 (PRDM16). White adipocytes can transdifferentiate into beige adipocytes through the action of diverse stimuli, including cold exposure which induce the expression of thermogenic-specific transcription factors, including PRDM16 and EBF2. From Cristancho et al. *Nat. Rev. Mol. Cell Biol*. 2011[41].

WAT depots initiate their development during late gestation, and adipogenic stimuli increases along with nutrient availability[66]. White adipocytes develop from PPARγ$^+$ αSMA$^+$ and PDGFRβ$^+$ progenitor cells that reside near the vasculature[66]. Bone morphogenetic proteins have been described to promote adipocyte terminal differentiation triggering PPARγ transcription. In addition, different zinc finger transcription factors have been implicated in the commitment of adipocyte differentiation, sensitizing fibroblasts towards pro-adipogenic BMP signalling and promoting the expression of master regulators of adipogenesis PPARγ and co- activator CCAAT/enhancer binding protein-α (C/EBPα)[65,67-69].

Even though white and brown adipocytes were thought to stem from a common precursor cell, fate-mapping studies have unveiled different progenitor cells for both white and brown adipocytes[70]. BAT development precedes WAT adipogenesis during embryogenic development, taking into account the urgent need of newborns to maintain body temperature through non-shivering thermogenesis[71]. *In vivo* lineage tracing provided evidence for a common embryonic precursor cell for brown adipocytes and skeletal muscle that transiently express the transcription factors *Pax7* and *Myf5*. The transcriptional regulator PRDM16 was found to determine the brown adipocyte phenotype[72,73]. The absence of PRDM16 in brown fat cells promotes myogenic differentiation. In fact, the methyltransferase EHMT1, an enzymatic component of the PRDM16 complex is critical to brown adipogenesis by repressing the activation of skeletal-muscle selective genes, enabling brown adipocyte differentiation[74].

Although beige adipocytes are characteristically similar to brown fat cells, these cells are developmentally distinct[73]. Similarly to white adipocytes, beige adipocytes are thought to derive from perivascular cells[75]. Even though the mechanisms of beige adipocyte maturation are still scarce, EBF2 and PRDM16 transcription factors are thought to play a role in beige adipogenic commitment, recruiting PPARγ to thermogenic gene targets, and initiating the adipogenesis of beige adipocytes[73,76]. Recently, ZFP423 has been documented to repress EBF2 transcriptional activity and to promote white adipocyte differentiation[77]. The brown-like adipocytes that develop within WAT depots in response to environmental cues may result from transdifferentiation of white adipocytes or from *de novo* adipogenesis from precursor cells.

Adipose Tissue Microenvironment and Cellular Crosstalk

Adipose tissue is a central metabolic organ compartmentalized in discrete depots and distributed throughout the human body. Adipocytes share their microenvironment and

interact with multiple cell types to coordinate adipose tissue functions. Vascular endothelial cells, sympathetic nerves, and immune cells compose the adipose tissue niche and modulate adipose function[78].

Angiogenesis

Angiogenesis and vascular regression are critical processes for adipogenesis. WAT remodelling is dependent on energy consumption and host metabolic demands, which also depend on nutrients and hormones transport to and from adipose tissue for maintenance of systemic energy demands, underlying the importance of the angiogenic machinery[58,79]. In addition, white adipocyte progenitors are also described to reside within the mural cell compartment of the involving adipose vessels[66]. Ingrowth of blood vessels in AT increases blood perfusion and provides enough oxygen supply to adipogenic processes. Upon expansion, adipocytes and adipose-derived stem cells within the adipose compartment secrete high levels of proangiogenic factors, including FGF-2, VEGF, HGF, ANGPTL4, PDGFs and leptin that sustain angiogenesis and contribute to energy homeostasis[58,80,81]. Relative to WAT, BAT is a highly vascularized tissue and express higher levels of VEGF. This translates into an increased angiogenic profile within BAT, that contributes towards thermogenesis by providing the necessary oxygen and substrate (fatty acids and glucose) to sustain the organism thermic control[44,58]. Consistently, VEGF has been documented to increase beige and brown fat development and its expression has been demonstrated to be upregulated upon cold exposure[82].

Innervation

Adipose tissue mass is intrinsically regulated by an endocrine loop that includes leptin release, which in turn acts upon neural circuits to regulate food intake and energy homeostasis[83]. Sympathetic innervation within adipose tissue regulates adipocyte function via the release of neurotransmitters, including brown adipogenesis and induction of adaptive thermogenesis[44,53]. Sympathetic nerve fibers density is directly correlated with beige fat adipogenesis, suggesting that beige fat development promotes nerve remodelling allowing for a more prominent thermogenic activation[44]. NGF and NRG4 are all adipocyte-released nerve remodelling factors that are thought to promote nerve growth, differentiation and branching[84]. In particular, the neurotransmitter norepinephrine, also known as noradrenalin, is both able to impact brown and white adipocytes, whether it induces

thermogenic processes or mediates leptin-dependent lipolysis for fatty-acid release, underlying the importance of AT innervation towards improved metabolic homeostasis[85].

Immune Regulation

Central to the metabolic homeostasis regulation is the immune compartment of the adipose tissue. Under normal conditions, adipose tissue is mainly composed of anti-inflammatory immune cell populations. M2-like macrophages secrete a diverse array of cytokines including IL-4 and IL-10 that contribute towards insulin sensitivity and adipogenesis. Eosinophils maintain this alternatively activated macrophages in the AT microenvironment by secreting type II cytokines such as IL-4 and IL-13[86]. Type II cytokine signalling increases with cold exposure and contributes to lipolytic processes, beige adipose tissue remodelling and BAT activation. Importantly, M2-like macrophages are an important source of catecholamines and are thought to play a critical role in WAT browning, typically less innervated than BAT, creating a sympathetic nerve-independent pathway towards thermogenic processes[87]. ILC2s and regulatory T cells (T_{regs}) also contribute towards beige fat activation and are responsible for eosinophil activation and survival through the secretion of IL-5. T_{regs} are beneficial to homeostatic regulation, promoting alternative macrophage activation and improving insulin sensitivity via PPAR-γ expression[88,89]. Curiously, phenotypically distinct T_{regs} were described in both WAT and BAT depots, implying depot-specific differences between different tissues[90,91]. Moreover, recent evidence suggests that WAT may function as a reservoir for memory T cells. Upon repeated infectious challenges, WAT suppresses lipid metabolic processes in favor of antimicrobial responses, allowing for long-term maintenance and rapid reactivation of memory T cells[92].

In the obese state, the hypertrophic adipocytes are more susceptible to inflammatory injury and cell death. The obese adipose tissue produces pro-inflammatory factors, including TNF, IL-6 and CCL2, recruiting monocytes that differentiate towards a pro-inflammatory state. In fact, adipose tissue macrophages (ATMs) can comprise up to 40-50% of all cells in obese individuals, contributing to the chronic inflammatory environment observed[93]. ATMs in obesity are polarized towards a pro-inflammatory state characterized by the expression of high levels of TNF and iNOS that contribute to metabolic disorder, impairing insulin sensitivity and inducing brown adipocyte apoptosis[44,94,95].

Adipose Secretory Profile and Metabolism

Prompted by the discovery of leptin and, a few years later, adiponectin, the physiology of adipose tissue highlights its role as an endocrine organ[96]. AT secretes a myriad of bioactive factors, termed adipokines, that contribute to the regulation of critical biological processes, including glucose maintenance, lipid, immune, and energy homeostasis[84]. These include white or brown adipocyte-derived secretory factors, including TNF, IL-6, FABP4, adiponectin, leptin, resistin, FGF21, NRG4, CCL2 and fatty acids[95,97,98].

Importantly, most of the above mentioned adipokines are upregulated in the obese state. The pro-inflammatory profile of obese individuals typically evolves to promote obesity-linked metabolic diseases. The upregulation of these pro-inflammatory adipokines (particularly TNF, IL-6, leptin, resistin and CCL2) contribute to the development of a chronic inflammatory state and, ultimately, to metabolic dysfunction.

Even though the alteration of the adipokine and lipid profile in response to nutritional cues are strongly associated with adipose tissue maladaptation, adiponectin is tightly associated with an anti-inflammatory profile of the healthy adipose tissue and increases free fatty acid oxidation and insulin sensitivity maintaining metabolic homeostasis. Contrarily, free fatty acid (including ceramides) ectopic deposition and spillover from adipocytes have lipotoxic effects, including mitochondrial dysfunction and insulin resistance. Notably, adiponectin, acting through its receptors AdipoR1 and AdipoR2, which possess ceramidase activity are thought to revert lipidic dysregulation and adipose tissue malfunction, decreasing ceramide levels within adipose depots. Arguably, leptin is the adipokine *per se* in the adipose compartment and is selectively expressed by adipocytes[99]. In healthy conditions, leptin has an essential role in suppressing appetite, energy expenditure and immune modulation. Therefore, it is not surprising that leptin-deficiency might lead not only to obesity, but also to an hyperglycemic condition and insulin-resistance[100,101]. Obesity usually increases the production of leptin. However, most of these individuals eventually develop leptin resistance, which is associated with elevated circulating levels of this adipokine and the inability of exogenously administrated leptin to decrease food intake and body weight, ultimately leading to a multitude of physiological alterations, including infertility, immune alterations and insulin resistance[83,102].

The contribution of the adipose tissue to homeostasis is remarkable. Minor alterations in the adipose tissue may lead to severe metabolic dysfunctions. Concordantly, it is invariably expected that adipose tissue also impacts cancer progression.

ADIPOSE TISSUE AND CANCER INTERFACE

Compelling evidence exist over the influence of the adipose tissue on cancer development. Excess adipose tissue accrual and AT dysfunction provides a unique microenvironment that favours both tumour initiation and progression. Even though increased adiposity is a well-established factor for cancer incidence and mortality, mechanistic insights that disclose the influence of this obese setting on cancer progression are currently scarce. Therefore, a better understanding of the processes through which adipose tissue expansion promotes tumourigenesis is critical to decrease obesity-related cancer incidence and improve treatment.

Epidemiologic Evidence for Obesity-Related Cancer

Obesity ensues a state of excessive accumulation of adipose tissue when caloric food intake largely exceeds energy expenditure. Excess adipose tissue is tightly associated with metabolic dysfunction and predisposition to several metabolic disorders, including type 2 diabetes mellitus and cardiovascular diseases. Ample large-scale epidemiological studies have unraveled a strong association between obesity and the risk of cancer development[103,104]. Obesity is estimated to contribute to approximately 9% of all cancers and 5-20% of all cancer-related deaths, promoting not only carcinogenesis, but the tumour development of established cancers as well[103,105]. In fact, evidence linking obesity to cancer development in the oesophagus, colon, kidney, pancreas, liver, thyroid, breast and endometrium is well established[106,107]. Intentional weight loss and/or bariatric surgery decrease cancer incidence, further supporting a role of excess adipose tissue in cancer[108]. At the same time, localized abdominal obesity has been specifically linked with cancer progression[109]. The interrelationship between the cellular and molecular pathways that are involved in obesity-associated tumourigenesis remain elusive. However, a number of mechanisms that link obesity with established and emerging hallmarks of cancer have been proposed, including inflammation, angiogenesis, immune regulation, EMT, genomic instability and cellular energetics[12,110].

Adipose Tissue Microenvironment and Cancer Progression

The survival of cancer cells is intricately related to its dependency on interacting with surrounding non-malignant tumour stroma cells. Adipose tissue within the tumour stroma actively contributes to tumour growth and metastasis providing an energetic reservoir for embedded cells. Signalling molecules secretion, including adipokines, proinflammatory and proangiogenic factors and ECM constituents exacerbate the importance of the AT as a critical contributor to cancer development and recurrence (**Figure 10**)[111,112].

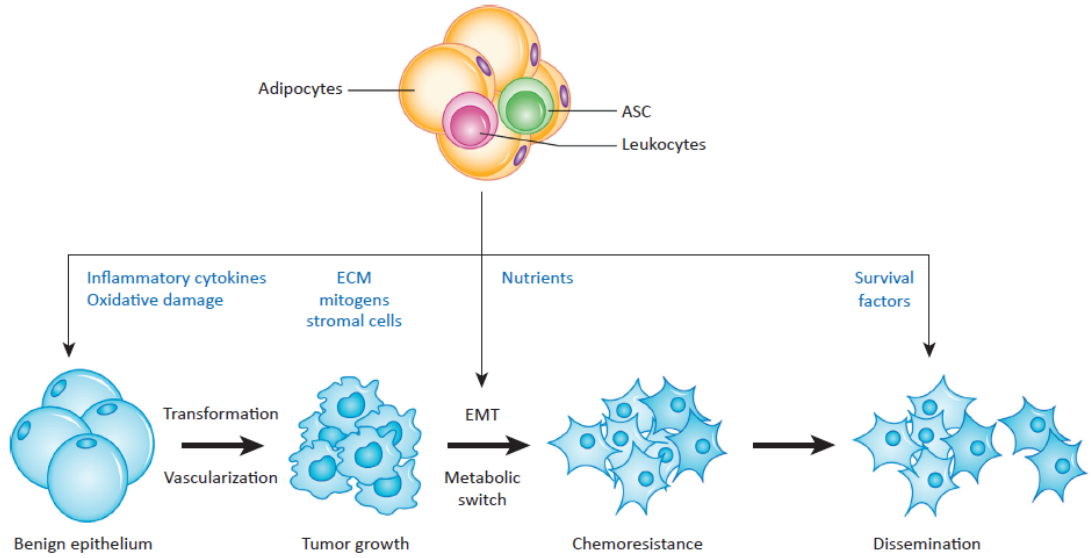


Figure 10 | **Adipose tissue promotes malignant transformation and tumour development**. Adipose tissue hypoxic hypertrophic expansion promotes the establishment of a chronic inflammatory state that may contribute to tumour initiation and progression. The adipose depot secretes a myriad of ECM constituents and growth factors that contribute to angiogenesis and mitogenic signalling that ultimately leads to tumour growth and invasive-associated features, including chemoresistance and dissemination. From Lengyel et al. *Trends Cancer* 2018[113].

Importantly, several features are shared between hypertrophic expansion of adipose tissue in the obese state and solid tumour growth. Tumour hypoxia is recurrently associated with worse outcomes for cancer patients and resistance to chemotherapy[114]. Concordantly, unbalanced adipose tissue expansion also induces hypoxia, triggering a series of compensatory angiogenic mechanisms to surpass nutrient and oxygen limited supply[58]. Similar to carcinogenic processes, profibrotic pathways are upregulated within the obese adipose microenvironment upon hypoxic conditions through the induction of HIF-1α-related pathways. Hypoxic adipose tissue upregulates the levels of ECM proteins (including collagens, matrix metalloproteinases and tissue inhibitors of metalloproteinases) and proinflammatory cytokines (such as TNF, IL-6 and CCL2), providing a tumour-permissive niche for transformed infiltrating cells (**Figure 11**)[115,116]. Notably, several proinflammatory and fibrotic molecules distinctive of obese individuals have been directly implicated in

tumour growth and metastasis. For instance, endotrophin, a C-terminal cleavage product of COL6α3 chain, has been reported to directly trigger adipose tissue fibrosis and metabolic dysfunction as well as promoting malignant tumour progression in mammary tumours[117,118].

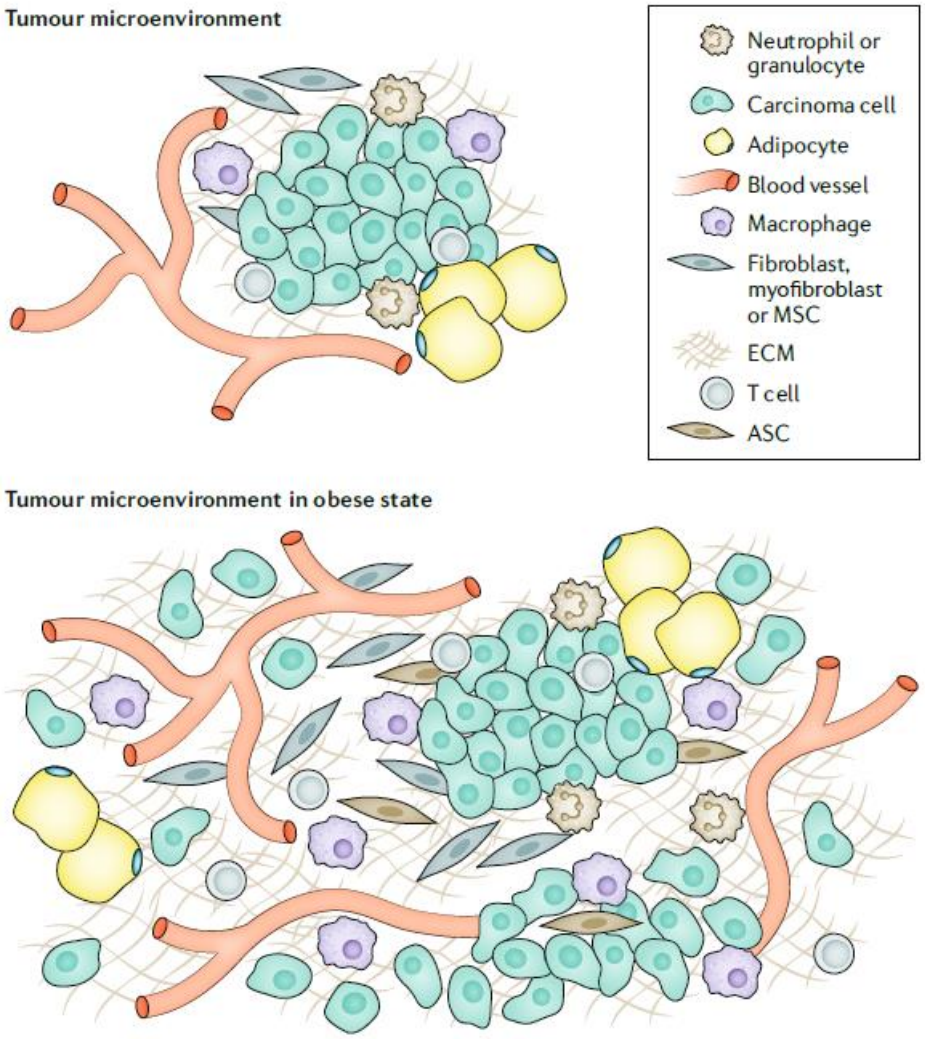


Figure 11 | **Obese tumour microenvironment**. During dysregulated hypertrophic growth of adipose tissue, the tumour niche is remodelled. Importantly, obesity promotes interstitial fibrosis altering ECM mechanotransduction. The tumour microenvironment within an obese state is enriched in adipose-derived stem cells that can into fibroblasts or adipocytes depending on environmental cues and tumour requirements to support tumour growth. In the obese-related cancer microenvironment the number of adipocytes is enriched, supporting tumour growth by providing tumour cells the fuel necessary to rapid cancer expansion. From O'Sullivan et al. *Nat. Rev. Gastroenterol. Hepatol.* 2018[104].

Inflammation as a Protumourigenic Mechanism

Unbalanced excess caloric intake and adipocyte hypertrophic growth leads to alterations in insulin sensitivity and cellular stress within fat cells given the surplus lipid accumulation and the oxidative stress, resultant from deficient neoangiogenic processes occurring within the obese state. The consequent hypoxic environment and lipid spillover

result in the release of inflammatory adipokines and in the increase on immune infiltrating cell rates, eventually leading to both local and distant chronic infllamation[119]. Chronic inflammation denotes a prolonged and dysfunctional protective mechanism that emerges in response to loss of tissue homeostasis, and has already been implicated as one of the hallmarks of cancer and underlying mechanistic process for tumourigenesis[12,120,121]. In lean individuals, adipose tissue immune infiltrate maintains tissue homeostasis, contributing to the clearance of apoptotic cells, angiogenic regulation and ECM remodelling, preserving a balanced microenvironment. In contrast, in obese subjects, immune cells often shift their phenotypic properties, promoting inflammation and consequent fibrosis[122]. The resulting fibrotic remodelling increases tissue stiffness and mechanotransduction, promoting carcinogenesis[123].

Even though AT-secreted molecules do not necessarily have mutagenic potential, it is plausible that the dysfunctional adipose tissue enhances mitogenic signalling to cells that already have carcinogenic mutations. However, genotoxicity is known to result from oxidative stress. The hypoxic AT microenvironment induces the production of reactive oxygen species (ROS) that, in turn, might cause DNA damage and impaired DNA repair[124,125]. In dysfunctional adipose tissue from obese patients, inflammatory signals recruit myeloid cells that apparently represent the major source of ROS within the AT, creating a chronic inflammatory state that ultimately conducts to genomic instability accrual and tumour initiating processes[126].

These processes are mainly elicited from adipocytes that prompt the adipose microenvironment through the secretion of a myriad of adipokines that modulate its environment and impact tumour development. However, not only adipocytes are responsible for the effects of this tissue on cancer initiation and progression[127]. The vascular stromal fraction of the AT, and in particular, the immune and adipose-derived stromal cells are critical players on adipose tissue landscape.

Adipose Immune Landscape and Cancer Cell Interactions

Adipose tissue contains a multitude of immune cells that contribute to cancer, from which macrophages are the most represented population[122]. These numbers increase in the setting of obesity, once adipocytes recruit and activate macrophages via CCL2 production, the major chemoattractant of monocytes to the obese tumour microenvironment[128]. AT-resident leukocytes may drive the tumourigenic process by systemically releasing an array of cytokines. It is also plausible that these cells are recruited

to the tumour microenvironment and act locally and/or at the AT-tumour invasive front. In fact, in obese individuals, AT-associated macrophages and tumour-associated macrophages are phenotypically and functionally similar, suggesting that AT macrophages may provide an identical activated microenvironment to that found in tumours[129,130]. Both populations induce fibrosis, angiogenesis and local hypoxia. The shift in the macrophage landscape from anti-inflammatory to proinflammatory leads to the above mentioned chronic inflammatory state and to alterations in lipid metabolism, which further contributes to cancer progression. In addition, a pool of cytokines released by these macrophages contributes to malignancy, promoting growth, ECM remodelling and tumour angiogenesis. The presence of aggregates of macrophages and of other immune cells around dead adipocytes, termed 'crownlike structures', is associated with increased aromatase levels and inflammation that potentiate cancer progression. Importantly, increased numbers of macrophages within breast cancer tumours are associated with high tumour grades and poor prognosis[131-133].

Even though only a few studies have dissertated over the influence of lymphocytes in the obese AT in cancer, these are undoubtedly important players in the TME. Notably, there is enrichment of activated and effector $CD4^+$ and $CD8^+$ T cells in the obese AT. These cells further exacerbate inflammatory macrophage recruitment and inflammatory cytokine production, including the secretion of MMPs, VEGF, TNF, IL-6 and CCL2, sustaining obesity-associated inflammation[122,134,135]. Importantly, obesity is thought to have a role in PD-1 regulation and immune tolerance. AT-associated macrophages expression of PD-L1 is upregulated by HIF-1α during hypoxia. At the same time, obesity promotes PD-1 expression in T cells, suppressing immunosurveillance and increasing immune tolerance[136,137].

Invariant natural killer cell (iNKT) numbers are usually increased in visceral adipose tissue of healthy humans. However, these cells are nearly absent from adipose tissue depots upon obesity. These cells are capable of recognizing lipid antigens, regulate adaptive immunity and are important players in the clearance of tumour cells[138]. However, iNKTs functional features and viability are impaired upon obesity and may further drive oncogenic processes[139].

Adipose-Derived Stem Cells

Adipocyte progenitors, termed adipose-derived stem cells (ASCs), are part of the stromal vascular fraction of the adipose tissue. This population is enriched within the obese setting and might be mobilized and recruited from adipose compartments to tumours to sustain cancer cell demands. CXCL1 and CXCL8 mediate the homing of ASCs to tumour

sites, where they may be incorporated into blood vessels as pericytes, or differentiate into multiple cell lineages, including adipocytes and cancer-associated fibroblasts[140-142]. Infiltrating ASCs within the tumour microenvironment directly impact cancer progression, driving tumour desmoplasia, recruiting macrophages and secreting proangiogenic factors, evidencing its tumour-promoting role[123,143]. ASCs have already been implicated in the growth, invasive potential and chemoresistance in ovarian and breast cancer[144,145]. Additionally, ASCs also contribute towards epithelial to mesenchymal transition during obesity-associated cancer by abnormally expressing leptin levels. Even though the exact mechanisms through which these cells are capable to impact tumour growth are still unresolved, the importance of ASCs crosstalk with cancer cells is undeniable, promoting cancer invasion and metastasis[146].

Tumour-Adipocyte Specific Interactions

Several types of carcinoma are known to invade adjacent adipose tissue. Therefore, it is not surprising that adipocytes directly interact with malignant cells in a bidirectional manner and contribute to tumour maintenance and progression[111,113]. In a subset of tumours, including breast, prostate, ovarian, colon, acute myeloid leukemia and renal cancers the adipocyte-rich microenvironment promote cancer growth and reflect a role for adipocytes in tumour development[111,112]. Notably, the crosstalk between adipocytes and cancer cells lead to considerable morphological and functional modifications: i. increases in adipocyte delipidation, ii. promotion of secretory proinflammatory molecules (including IL-6, IL-8 and PAI-1) and, iii. decrease in adipocyte terminal differentiation markers. Therefore, cancer associated adipocytes (CAAs) become tumour-educated and acquire a fibroblast-like and cancer-promoting phenotypes (**Figure 12**) [147-149].

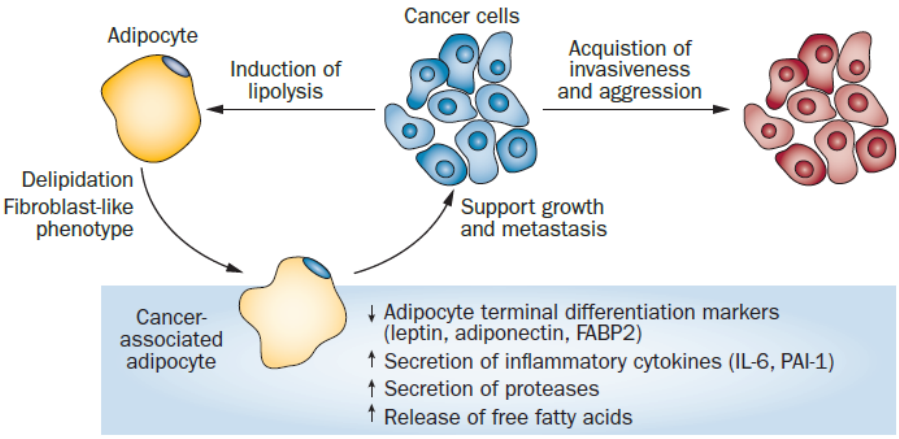


Figure 12 | **Cancer cells and cancer-associated adipocytes crosstalk**. The interaction between cancer-associated adipocytes (CAAs) and cancer cells promotes the creation of a niche that is permissive for tumour growth and metastasis. Cancer cells stimulate adipocytes delipidation and the acquisition of a fibroblast-like phenotype. CAAs decrease adipocyte terminal differentiation markers and increase the secretion of inflammatory cytokines and proteases that increase tumour progression and invasive potential. The rapid growth of tumour cells is accompanied by the release of free fatty acids by CAAs that support tumour growth and provide the necessary energy to fuel tumour development. From Park et al. *Nat. Rev. Endocrinol.* 2014[112].

Cellular Energetics and Metabolic Symbiosis - Beyond the Warburg Effect

Tumour cells often shift their metabolic status, generating ATP via glycolytic processes rather than oxidative phosphorylation even under aerobic conditions[12]. Emerging insights over the metabolic features of cancer cells have suggested that aerobic glycolysis occurs in tumour surrounding cells rather than in cancer cells itself, providing to malignant cells the necessary nutrients, via a paracrine exchange, to generate energy by mitochondrial oxidative metabolic processes[150,151]. CAAs survival-promoting effect on tumours are increasingly being associated with metabolic symbiosis, suggesting that adipocytes within the TME provide nutrients to cancer cells, sustaining tumour growth and survival[152-155].

With hypoxia, occurring metabolic reprogramming in cancer cells increase lipid usage, associated with cancer aggressiveness. The primary source of lipids used by cancer cells are fatty acids (FAs). In fact, FAs utilization is a more efficient way to produce energy through β-oxidation since it allows cells to increase energetic yields relatively to glucose[156,157]. Importantly, FAs derivatives are important are important components of cancer cell structure and mitochondrial membrane, further supporting tumourigenesis[158]. Transformed malignant cells are capable of synthesizing FAs *de novo* and abnormally elevated lipogenesis is a hallmark of aggressive and metastatic cancers[159,160]. However, in rapid growing and more aggressive cancers, endogenous lipogenesis becomes limiting and

tumour cells increase the uptake of extracellular FAs[161]. Cancer associated adipocytes hydrolyze triglycerides to release free FAs and consequent uptake by malignant cells. In this regard, FABP4, a FA-binding protein, mediates the trafficking of adipocyte-derived FAs between CAAs and cancer cells. In turn, transformed cells upregulate the levels of CD36, which facilitates FA uptake, enabling the usage of FAs as a substrate for β-oxidation, providing the tumour with sufficient energy requirements for growth and progression[153-155]. Notably, in the obese setting, increased levels of systemic adipocyte-derived FABP4 promotes mammary tumour stemness and aggressiveness[162]. Moreover, overexpressing CD36 cells were demonstrated to boost the metastatic potential of oral squamous cell carcinomas[163]. Therefore, adipocyte-derived FAs might constitute an important substrate to support tumour growth and metastasis and may provide novel therapeutic approaches to treat different cancers.

Focus on Leptin and Adiponectin

Leptin is an adipocyte-derived hormone that regulates appetite and energy homeostasis[46]. Leptin levels are intrinsically correlated with increased adiposity in humans, and studies have associated this hormone with increased cancer incidence in obesity. Leptin signals through a transmembrane receptor (OBR) that mediates intracellular signalling through STAT3, increasing pro-inflammatory signalling within the cell, which promotes cancer progression[164]. Moreover, leptin functions to promote mitogenic effects, to mediate immune suppression and to support angiogenesis[165]. Furthermore, leptin is thought to induce telomerase reverse transcriptase expression, EMT and stemness[166-168]. In summary, leptin may promote tumour progression.

Adiponectin is an adipokine with pleiotropic actions, including insulin-sensitizing, anti-inflammatory and anti-apoptotic functions, and regulates multiple processes, including glucose and lipid metabolism[169]. In the obese setting, adiponectin levels are negatively correlated with adipose mass, and this decrease is associated with cancer aggressiveness[164,170]. Both indirect and direct effects of adiponectin in cancer progression have been elucidated. Adiponectin binding to its receptors (ADIPOR1 and ADIPOR2) sequesters growth factors, activating AMPK signalling while impairing several other intracellular mediators (including ERK1 and ERK2, PI3K-AKT, WNT-β-catenin and STAT3 signalling), which negatively impacts fatty acid and protein synthesis, cellular growth and proliferation, while enhancing genomic toxicity, increasing cell cycle arrest and apoptosis. Therefore, adiponectin appears to have a protective role in carcinogenesis[171,172].

ADIPOSE TISSUE AT THE NEXUS OF RENAL CELL CARCINOMA

Obesity pandemics is a mounting health concern worldwide and is associated with increased risk to develop multiple types of cancer, including renal cell carcinoma. RCC comprises over 90% of all kidney cancers and is tightly associated with excess adiposity. In fact, a 5 kg/m^2 increase in body mass index (BMI) is strongly correlated with the risk to develop RCC[106]. Tumours invade stromal compartments that are enriched in adipose tissue, and the ensuing crosstalk shapes the tumour microenvironment and contributes to cancer progression[173]. Importantly, several reports indicate towards a counterintuitive premise, known as the 'obesity paradox': even though excess adiposity is associated with RCC development, obese patients, defined as a BMI ≥ 30, had a median overall survival superior to lean individuals[174-178]. Moreover, even though the mechanisms that contribute to this 'obesity paradox' are largely unknown, different studies have pointed towards a role of decreased adiponectin[175] allied to an increase in FASN[174] levels in obesity-related cancer-specific survival.

Currently, mechanisms linking obesity with RCC incidence are mainly unknown. Studies performed on diet-induced obesity animal models illustrated a relationship between the obese adipose compartment and renal cancer, where obesity promotes an immunosuppressive environment, increasing myeloid-derived suppressor cells accumulation and impairing dendritic cell response to RCC[179,180]. Moreover, cell line-based studies unveiled an effect of perirenal adipose tissue (PrAT) on migration and invasive potential of RCC, and propose a potential role of WNT signalling on disease progression[181,182].

Although only a few studies have disserted over the influence of the AT in RCC, a clearer picture of the crosstalk between both tissues is underway. However, further studies are in need to fully disclose the role of the adipose organ in cancer and, particularly, in renal cell carcinoma.

AIMS

The interrelationship between clear cell renal cell carcinoma (ccRCC) and surrounding adipose tissue (AT) is still very scarce. This project sets to provide insight over the ensuing crosstalk between clear cell renal cell carcinoma and the adipose tissue, focusing on the nexus between the perirenal and subcutaneous adipose depots and ccRCC. Several studies portray the impact that different adipose tissues have on distinct types of cancer. The ccRCC/AT interface is of particular importance since, even though obesity increases the relative incidence of this type of cancer, obese patients have superior overall survival rates than its counterpart lean individuals. To address this issue, this project sets its basis on three main specific aims:

1. To give a comprehensive characterization of both perirenal and subcutaneous adipose depots, establishing the distinct features of both ATs and assess its influence on clear cell renal cell carcinoma.
2. To evaluate the influence of adipose tissue-derived secreted factors on clear cell renal cell carcinoma genetic signature.
3. To address the role of adipose tissue in the functional activity of clear cell renal cell carcinoma cells, specifically, proliferative and apoptotic processes.

The conclusions drawn from this work will increase the amount of growing evidence that interconnects the adipose depot with clear cell renal cell carcinoma and sets some groundbreaking insights to potentiate novel research and therapeutic strategies that target adipose function in the treatment of ccRCC.

MATERIALS AND METHODS

Clinical Samples and Adipose Tissue *Ex Vivo* Culture

Human perirenal (PrAT) and subcutaneous (ScAT) adipose tissue samples were collected from renal cell carcinoma patients undergoing radical or partial nephrectomy in accordance with the recommendations of the ethics committee of the participating Hospitals (Centro Hospitalar do Porto e Centro Hospitalar do Alto Ave). All patients gave their informed consent to participate in this study, which was conducted according to the Declaration of Helsinki Principles. PrAT and ScAT samples were collected, transported in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Invitrogen) supplemented with 10% of newborn calf serum (NBCS) and immediately processed. Tissue was dissected to remove major vessels and further parted into 5-10 mm pieces and washed several times with PBS upon processing. Explants were cultured for 16h in DMEM/F12 supplemented with 10% of NBCS (0.3 g of explant/ml medium) and incubated at 37ºC, 5% CO_2. Following the 16h of culture, the medium was renewed and fresh non-supplemented DMEM/F12 was added. Explants were maintained at 37ºC, 5%CO_2. After 48 hours, the undernatant was collected, centrifuged (5 min, 1500 g), aliquoted and stored at -80º as explant conditioned medium (CM).

Cell Lines Maintenance and Culture Conditions

786-O and Caki-1 RCC cell lines (**Table 1**) were cultured in RPMI-1640 (Gibco, Invitrogen) and McCoy's 5A (Gibco, Invitrogen) media, respectively. Media were supplemented with 1% penicillin-streptomycin (Pen/Strep) (Biowest, 100x, cat no. L0022-100) and 10 % fetal bovine serum (FBS) (Biowest, cat. Nº S181BH-500). Cell lines were maintained at 37ºC in a humidified atmosphere of 5% CO2. The cells were detached with 0.05% trypsin (Sigma-Aldrich, T4799) for 5- 10 minutes at 37ºC and its action was neutralized using RPMI-1640 or McCoy's 5A medium containing 1% Pen/Strep (Biowest) and 10% FBS (Biowest). The cells were used for assays when >80% confluence was reached.

Table 1 | **Clear cell renal cell carcinoma lines.**

Cell Line	*VHL* Status	Derived From
786-O	Inactivating mutation	Primary Tumour
Caki-1	Wild Type	Skin Metastasis

Adipocyte Size Measurements

Adipocyte area size was determined from H&E stained paraffin sections of both perirenal and subcutaneous adipose tissue. Cross-sectional area of adipocytes was obtained from perimeter tracing of cells using ImageJ. At least 100 adipocytes were quantified for each patient.

Cell Proliferation

786-O (1.5×10^4 cells/well) or Caki-1 (2.0×10^4 cells/well) cell lines were seeded into 6-well plates in complete RPMI-1640 or McCoy's 5A complete media, respectively, and cultured for 24 hours at 37ºC, 5% CO_2. Following incubation period, media was removed, and cells were washed with PBS three times and the medium renewed with RPMI-1640 or McCoy's 5A complete media with (50% volume) or without (control) adipose tissue-derived conditioned medium. Cell lines were maintained at 37ºC in a humidified atmosphere of 5% CO2. 24 hours later, cells were detached and stained with EdU using Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Invitrogen, cat no. C10634) according to manufacturer's instructions. Following staining, samples were filtered, and cells were acquired on a FACSCanto flow cytometer (BD Biosciences) using FACSDiva software. Data analysis was performed with FlowJo software.

Apoptosis Assay

786-O (1.5 x 10^4 cells/well) or Caki-1 (2.0 x 10^4 cells/well) cell lines were seeded into 6-well plates in complete RPMI-1640 or McCoy's 5A complete media, respectively, and cultured for 24 hours at 37ºC, 5% CO_2. Following incubation period, media was removed, and cells were washed with PBS three times and the medium renewed with RPMI-1640 or McCoy's 5A complete media with (50% volume) or without (control) adipose tissue-derived conditioned medium. Cell lines were maintained at 37ºC in a humidified atmosphere of 5% CO2. 24 hours later, cells were detached and stained with FITC Annexin V and propidium iodide (PI) using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, cat no. 556547) according to manufacturer's instructions. Following staining, samples were filtered, and cells were acquired on a FACSCanto flow cytometer (BD Biosciences) using FACSDiva software. Data analysis was performed with FlowJo software.

Zymography

Conditioned media from perirenal and subcutaneous adipose tissues were analysed by gelatin zymography. Protein content was determined using the DC protein assay kit (BioRad), and 15 µg of protein were mixed with a non-reducing sample buffer (10% SDS, 4% sucrose, and 0,03% bromophenol blue in 0.5 M Tris-HCl pH 6.8) and PBS. Separating (10% polyacrylamide, 0.1% gelatin (Bovine skin type B, Sigma G9391) and stacking (2,5% polyacrylamide) gels were prepared. The samples were loaded and separated for approximately 4 hours at 80V. After electrophoresis, gels were washed twice with 2% Triton X-100 for protein renaturation. The gels were incubated for 16 hours at 37 ºC in MMP substrate buffer (50 mM Tris-HCl; pH 7.5, 10 mM CaCl2 and 0,5 % NaN3). After the incubation, gels were stained with Coomassie Blue solution (Sigma Aldrich) for approximately 30 minutes followed by a wash with deionized water. White bands of proteolytic degradation appeared on a blue background. Finally, the gels were scanned and the MMPs molecular weight and activity was estimated by densitometric analysis (QuantityOne, BioRad).

RNA Extraction and Purification

Adipose Tissue

Perirenal and subcutaneous adipose tissue were preserved in RNAlater RNA Stabilization Solution (Invitrogen, cat no. AM7020). Total RNA was extracted from 100 mg of tissue was lysed in 1.2 mL of QIAZOL Lysis Reagent and homogenized with TissueRuptor II (QIAGEN, cat no. 9002756), using disposable probes. RNA was purified using the RNeasy Lipid Tissue Mini Kit (QIAGEN, cat no. 74804) according to manufacturer's instructions.

Cell Lines

Total RNA was extracted from the cells previously cultured in perirenal- or subcutaneous AT-derived CM. After detachment, cells were centrifuged at 1200 rpm for 5 minutes at 4ºC, resuspended in 500 µL of TriPure Isolation Reagent (Roche, ref. 11667165001) in a fume hood and according to the manufacturer´s instructions, and transferred into 1,5 mL eppendorf tubes. The cells were then stored at -80º C until further use. For the RNA extraction, the cells were incubated for 5 minutes at RT to ensure the complete dissociation of nucleoprotein complexes. After incubation, 100 µL of chloroform were added to each sample and the tubes were shaken vigorously for approximately 15 seconds. The tubes were then incubated at RT for 15 minutes and centrifuged at 12000g for 15 minutes at 4ºC in order to separate the solution into three phases. After the centrifugation, the colorless upper part of the solution was transferred into new tubes. In order to precipitate the RNA, isopropanol was added (250 µL per tube) and the tubes were gently inverted manually, followed by an incubation at RT for 10 minutes. Afterwards, the tubes were centrifuged at 12000g for 10 minutes at 4ºC and the supernatant was discarded. Ethanol was added (500 µL per tube) and the RNA was washed by vortexing, and then centrifuged at 7500g for 5 minutes at 4ºC. The supernatant was discarded and the excess ethanol from the RNA pellet was removed by air drying for at least one hour. Subsequently, each pellet was resuspended in 20 µL of RNAse-free water and incubated for one hour at 4ºC. RNA concentration and purity were analysed using a Nanodrop Spectrophotometer ND1000 (Thermo Scientific). The RNA samples were stored at -80ºC until further use.

Complementary DNA First-Strand Synthesis

The synthesis of cDNA was performed using 400 ng (for adipose tissue) or 1 µg (for cell lines) of RNA. Two independent mixes were prepared. The first mix (annealing mix) was prepared with 1 µg of the previously extracted RNA, 1 µL of random primers and 9 µL of RNAse/DNase free water. The mix was then incubated for 10 minutes at 70 ºC using a thermocycler (BIOMETRA T1 Thermoblock Thermal Cycler). The second mix was composed of 4 µL of 5x first-strand buffer (Invitrogen), 1 µL of 10 mM dNTPs Mix (Invitrogen) 2 µL of 0,1 mM DTT (Invitrogen), 0,2 µL of NZY Ribonuclease Inhibitor (nzytech), 0,5 µL 52 of Reverse Transcriptase (nzytech) and 1 µL of RNAse/DNase free water (Invitrogen). After the incubation of the first mix, 8 µL of the second mix was added to the annealing mix and incubated for 1 hour at 37ºC using the previously mentioned thermocycler. The obtained cDNA was stored at -20º until further use.

Quantitative Real-Time PCR

After the cDNA synthesis, qRT-PCR was carried out using a TaqMan Universal PCR Master Mix (KAPA PROBE FAST qPCR Master Mix, lKAPA Biosystems) and probes for *UCP1*, *PRDM16*, *EVA1*, *TBX1*, *TMEM26*, *CD137*, *SLC7A10*, *HIF1α*, *HIF2α*, *IL6*, *TGFβ1*, *SNAIL*, *SLUG*, *ZEB1*, *ZEB2*, *TWIST1*, *OCT4*, *NANOG* and *TBP* or *GAPDH* (used as housekeeping genes for AT and cell lines, respectively) (**Table 2**). The qRTPCR was performed in a 7500 Real Time PCR system (Applied Biosystems). The program was 1 step of 95 ºC for 20 seconds followed by 40 cycles of 95ºC for 3 seconds and 60 ºC for 30 seconds. Each reaction was executed in triplicate and relative mRNA expression of the target genes was normalized to the levels of the housekeeping gene using the comparative ΔΔCT method.

Table 2 | **Gene assays for qRT-PCR**. All probes used were supplied by Integrated DNA Technologies (IDT)

Gene	Assay ID	Gene	Assay ID
UCP1	Hs.PT.58.39157006	*TGFβ1*	Hs.PT.58.39813975
PRDM16	Hs.PT.58.25688289	*SNAIL*	Hs.PT.58.2984401
EVA1	Hs.PT.58.996348	*SLUG*	Hs.PT.58.1772559
TBX1	Hs.PT.58.2672803	ZEB1	Hs.PT.58.39178574
TMEM26	Hs.PT.58.4285425	ZEB2	Hs.PT.58.1089006
CD137	Hs.PT.58.40744808	*TWIST1*	Hs.PT.58.18940950
SLC7A10	Hs.PT.58.25903360	*OCT4*	Hs.PT.58.14648152.g
HIF1α	Hs.PT.58.534274	*NANOG*	Hs.PT.58.21480849
HIF2α	Hs.PT.58.2273374	*TBP*	Hs.PT.58.20792004
IL6	Hs.PT.58.40226675	*GAPDH*	Hs.PT.39a.22214836

Statistical Analysis

All the data obtained in this work was organized and analysed using the GraphPad Prism 6 software. Data were analyzed for Gaussian distribution using D'Agostino-Pearson or Shapiro-Wilk normality tests. Statistical tests are registered in the figures' captions. The significance was defined as $P \leq 0.05$.

RESULTS

ADIPOSE TISSUE CHARACTERIZATION

Renal cell carcinoma malignant cells may influence the surrounding adipose tissue to acquire phenotypic features that benefit tumour growth. Furthermore, cancer cells influence distant niches in order to promote metastatic behaviour. In this work, two distinct adipose depots were used to understand the role of depot-specific influences on ccRCC. Notably, and even if the peritumoural adipose tissue is of crucial importance in the tumour landscape, the usage of a different distant depot, as the subcutaneous AT, to understand the relative influence of individual fat pads in ccRCC is mandatory. It is of utter importance to, firstly, characterize the different depots to assess the existence of intrinsic differences between perirenal (PrAT) and subcutaneous (ScAT) adipose tissues. A characterization of the above-mentioned depots will be conceded in this section, which will comprehend morphological, genetic and functional analysis of the distinct adipose repertoires that will constitute the foundation for the rest of this work.

Perirenal and Subcutaneous Adipose Tissue Have Distinct Morphological Features

To assess the differences between perirenal (PrAT) and subcutaneous (ScAT) adipose depots, the morphological appearance of PrAT was compared to that of ScAT (**Figure 13**). Hematoxylin and eosin (H&E)-stained paraffin-embedded sections of both adipose depots were analysed regarding its morphological aspect (**Figure 13A**). No apparent differences could be observed between PrAT and ScAT. Both depots presented an unique large unilocular lipid droplet occupying most of the cell cytoplasm. However, visual differences could be detected when comparing the adipocyte size between both tissues (**Figure 13A**). In fact, microscopic determination of the cross-sectional area of individual adipocytes from the two different tissues revealed a significant difference between PrAT and ScAT. These measurements demonstrated an increased size regarding PrAT adipocytes in comparison to ScAT fat cells (**Figure 13B**).

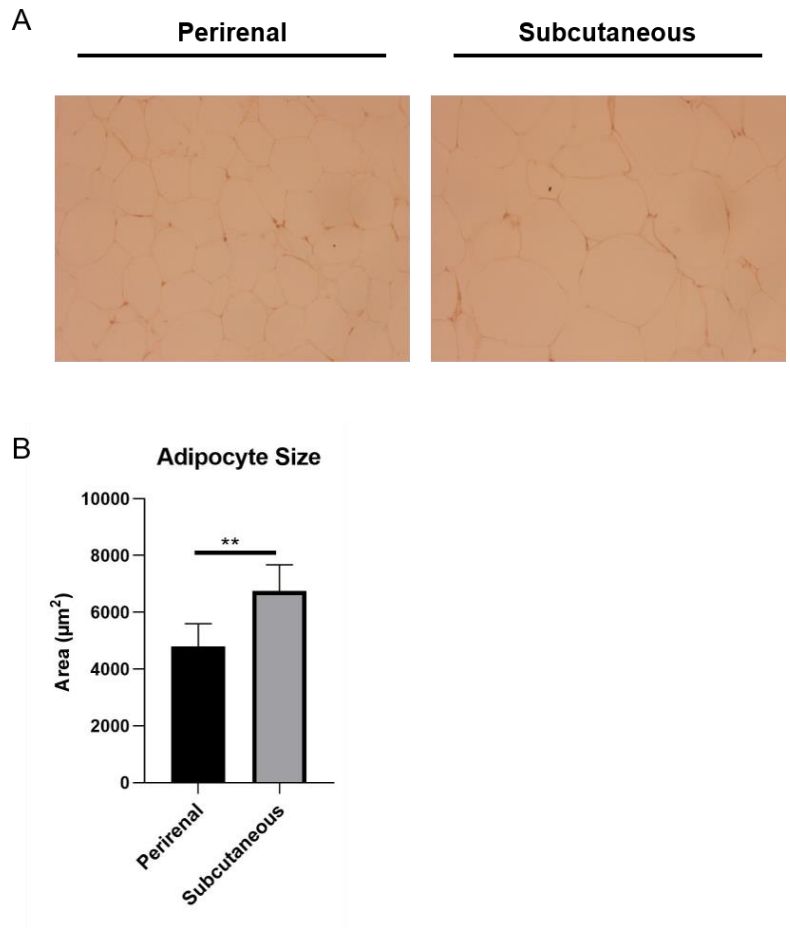


Figure 13 | **Perirenal and subcutaneous adipocytes have distinct sizes**. H&E stained paraffin embedded sections from perirenal (PrAT) and subcutaneous (ScAT) adipose tissues collected from renal cell carcinoma (RCC) patients submitted to partial or radical nephrectomy were analysed. (A) Representative images of H&E cross-sectional sections (200x ampliation) of both perirenal (left) and subcutaneous (right) adipose depots. (B) Microscopic quantification of individual adipocytes area in PrAT (n=7) and ScAT (n=6) of the aforementioned H&E-stained sections. Bars represent mean values and error bars represent standard deviation (SD). **$p < 0.01$ as determined by unpaired two-tailed Student's t test.

In summary, morphological analysis disclosed the first evidence for distinct site-specific features between PrAT and ScAT in this work.

Perirenal Adipose Tissue Displays a Trend to Increase Thermogenic-Selective Genes

Following adipose morphological characterization, a genetic-based approach of a panel of genes known to be differentially express in white, brown and beige adipocytes was conducted. RNA extracted from perirenal and subcutaneous adipose tissues collected from RCC patients that were submitted to partial or radical nephrectomy was used to assess relative mRNA expression levels of *UCP1*, *PRDM16* and *EVA1* (brown-selective genes); *CD137*, *TMEM26* and *TBX1* (beige-selective genes); and *SLC7A10* (white-selective gene) (**Figure 14**).

Overall, the levels of brown-selective genes underlined a trend for PrAT to upregulate the expression of these genes when compared to ScAT (**Figure 14**, upper panel). Importantly, even though not statistically different, *UCP1* gene expression levels were higher in all PrAT samples than in ScAT counterparts (data not shown). However, no differences could be seen regarding to *PRDM16*.

Whereas *CD137* levels tended to increase in ScAT, *TMEM26* observed expression was the opposite. *TMEM26* presented increased levels in PrAT when compared to ScAT. No major differences could be observed when comparing the mRNA expression levels of *TBX1* between both depots (**Figure 14**, lower panel, left).

Even though the levels of brown-like genes were relatively increased in PrAT, this tissue significantly increased *SLC7A10* expression levels compared to ScAT (**Figure 14**, lower panel, right).

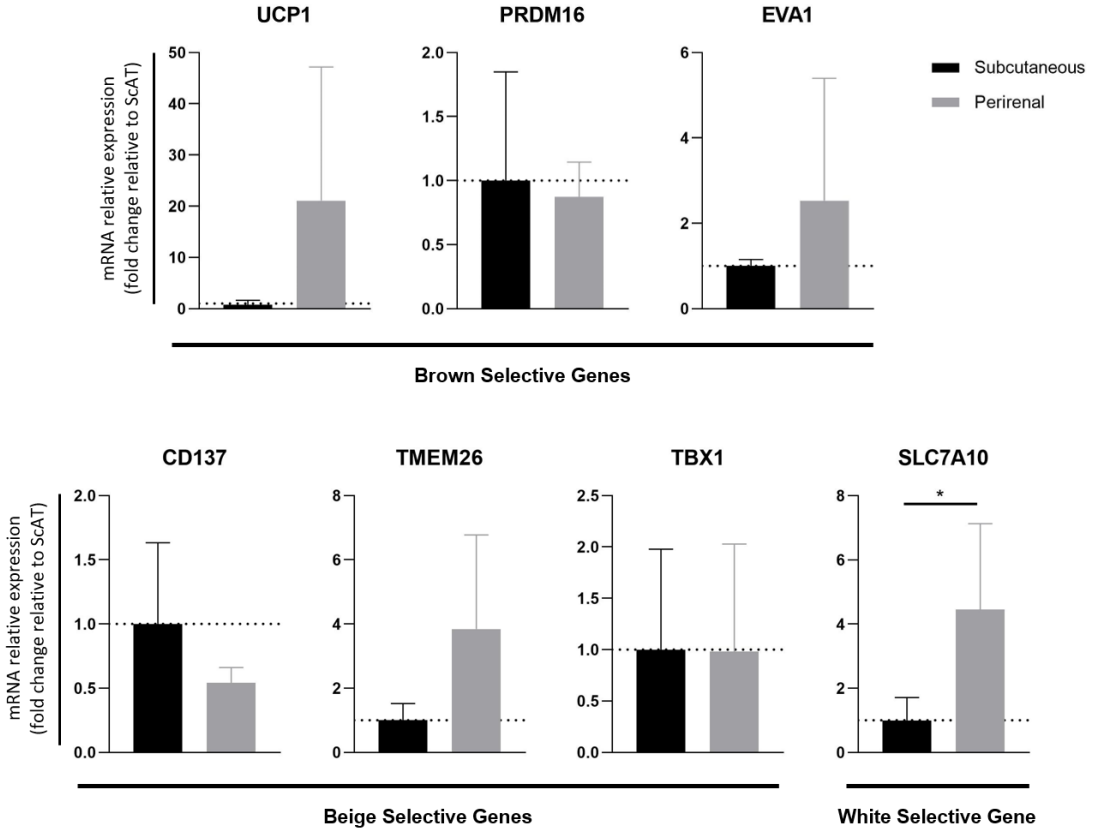


Figure 14 | **Gene expression profile of perirenal (PrAT) and subcutaneous (ScAT) adipose depots.** RNA extracted from paired PrAT and ScAT collected from RCC patients submitted to partial or radical nephrectomy was reverse transcribed and analysed by quantitative real-time PCR (qRT-PCR). A panel of marker genes for brown (*UCP1*, *PRDM16* and *EVA1*), beige (*CD137*, *TMEM26* and *TBX1*) and white (*SLC7A10*) adipocytes were assessed and its mRNA levels determined (n=5). Relative gene expression was normalized to TATA box-binding protein (*TBP*) mRNA levels. Bars represent mean values and error bars represent standard deviation (SD). *p < 0.05 as determined by paired two-tailed Student's t test.

Altogether, PrAT collected from RCC patients evidenced a thermogenic gene program signature, given the trend to enhance the expression of both brown- and beige-selective genes, when compared to ScAT, even though higher levels of the white-selective gene, SLC7A10, could also be found in this depot.

Perirenal Adipose Depot Secretes Higher Amounts of Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are proteolytic enzymes that have long been associated with cancer cell invasive and metastatic potential through ECM remodelling, and the regulation of tumour angiogenesis and immune surveillance[183]. In fact, MMPs secreted by peritumoural adipose depots have already been described to promote tumour cell invasive capabilities[148]. To functionally characterize the different adipose depots, gelatin zymography studies were performed to assess MMPs activity on the supernatants of PrAT and ScAT *ex vivo* explant cultures (**Figure 15**).

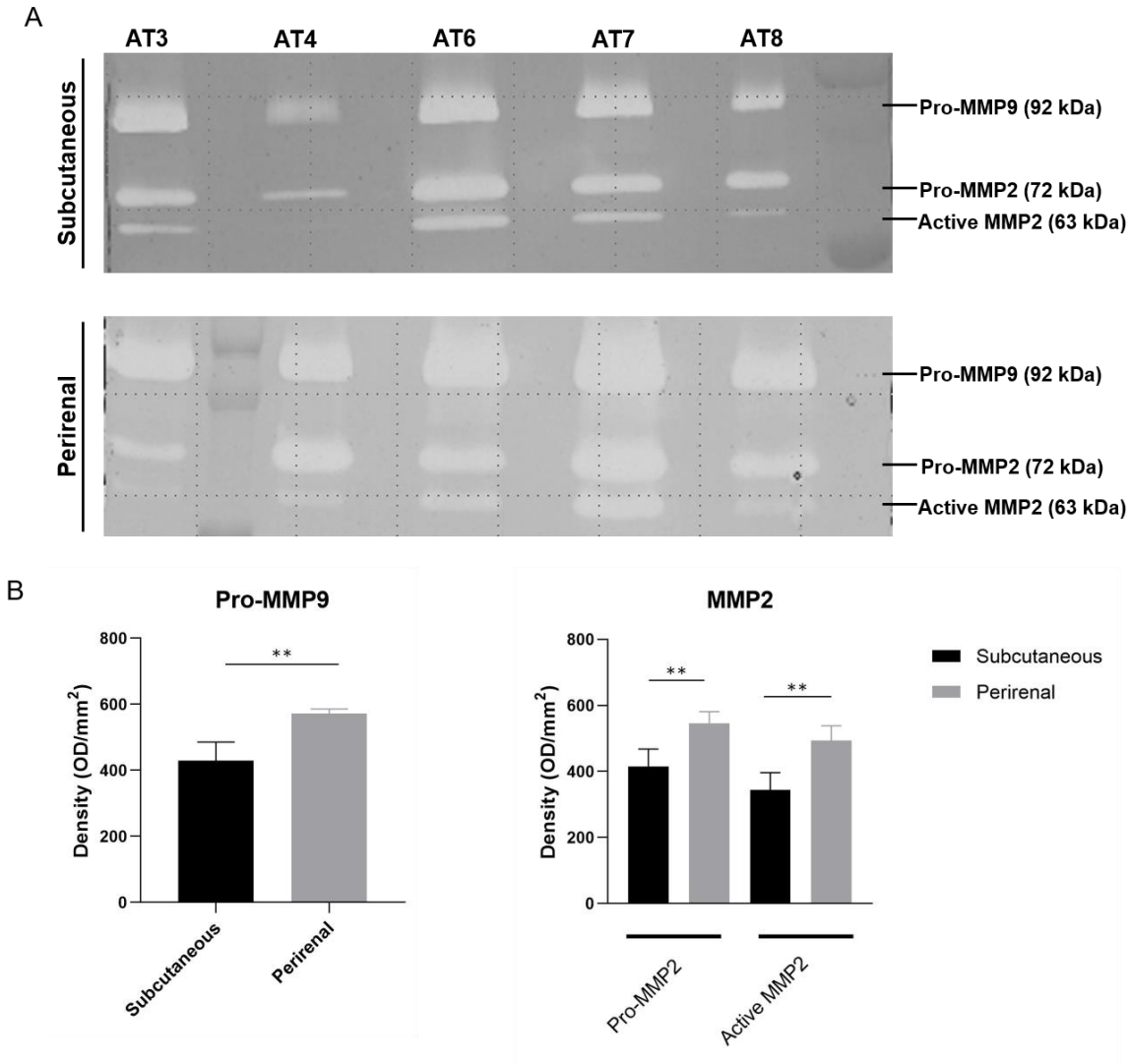


Figure 15 | **MMP2 and MMP9 proteolytic activity in perirenal- and subcutaneous-derived conditioned media**. (A) Conditioned media (CM) collected from the *ex vivo* culture of perirenal and subcutaneous adipose tissue were run on gelatin zymograms. Total protein of the different CMs was quantified and the same amount of protein was loaded. Proteolytic activity was revealed as white bands on a dark background stained with Coomassie. (B) Densitometry analysis of paired adipose depots derived from 5 different patients, as given by the optical density (OD) per mm^2, was performed using Quantity One (Bio-Rad). Data are represented as mean ± SD. **$p < 0.01$ as determined by paired two-tailed Student's t test.

Overall, **Figure 15A** depicts an increased activity of MMP2 (both pro and active forms) and pro-MMP9 in PrAT-derived conditioned media (CM) relative to ScAT-derived CM. In fact, data obtained from the densitometry analysis of the proteolytic activity of both AT-derived supernatants revealed a significant increase in the activity of the all set of MMPs analysed in the perirenal depot in regard to subcutaneous adipose tissue (**Figure 15B**).

Collectively, these data indicate that, in fact, intrinsic differences between perirenal and subcutaneous adipose depots exist. In summary, perirenal displays smaller adipocytes, exhibits increased expression of thermogenic-related genes and enhanced secretion of matrix metalloproteinases, evidencing that site-specific differences may contribute differently to clear cell renal cell carcinoma.

ADIPOSE TISSUE AND CLEAR CELL RENAL CELL CARCINOMA CROSSTALK

Obesity has been largely associated with the incidence of clear cell renal cell carcinoma, underlying a clear effect of the hypertrophic adipose depots to cancer promotion and progression. To better understand the influence of different adipose depots in cancer development, 786-O and Caki-1 cell lines cultured under the influence of PrAT- or ScAT-derived CM of paired patients were used. An initial approach focused on a comprehensive analysis on the cellular-specific gene expression modifications and, at a later stage, cells were submitted to functional analyses in order to understand if changes in the gene expression profile of these cells translated into a physiological mechanism.

Adipose Tissue Alters the Gene Expression Profile of 786-O Cells

Following *ex vivo* explant culture of perirenal and subcutaneous adipose tissue explants, the resulting supernatants were collected, and 786-O cells were cultured with or without (control, CTRL) the influence of these conditioned media. To examine whether soluble factors secreted by the different adipose depots alter 786-O gene expression, RNA extracted from cells stimulated or non-stimulated with AT supernatants was used to assess relative expression levels of genes associated with tumour-promoting processes (**Figure 16**).

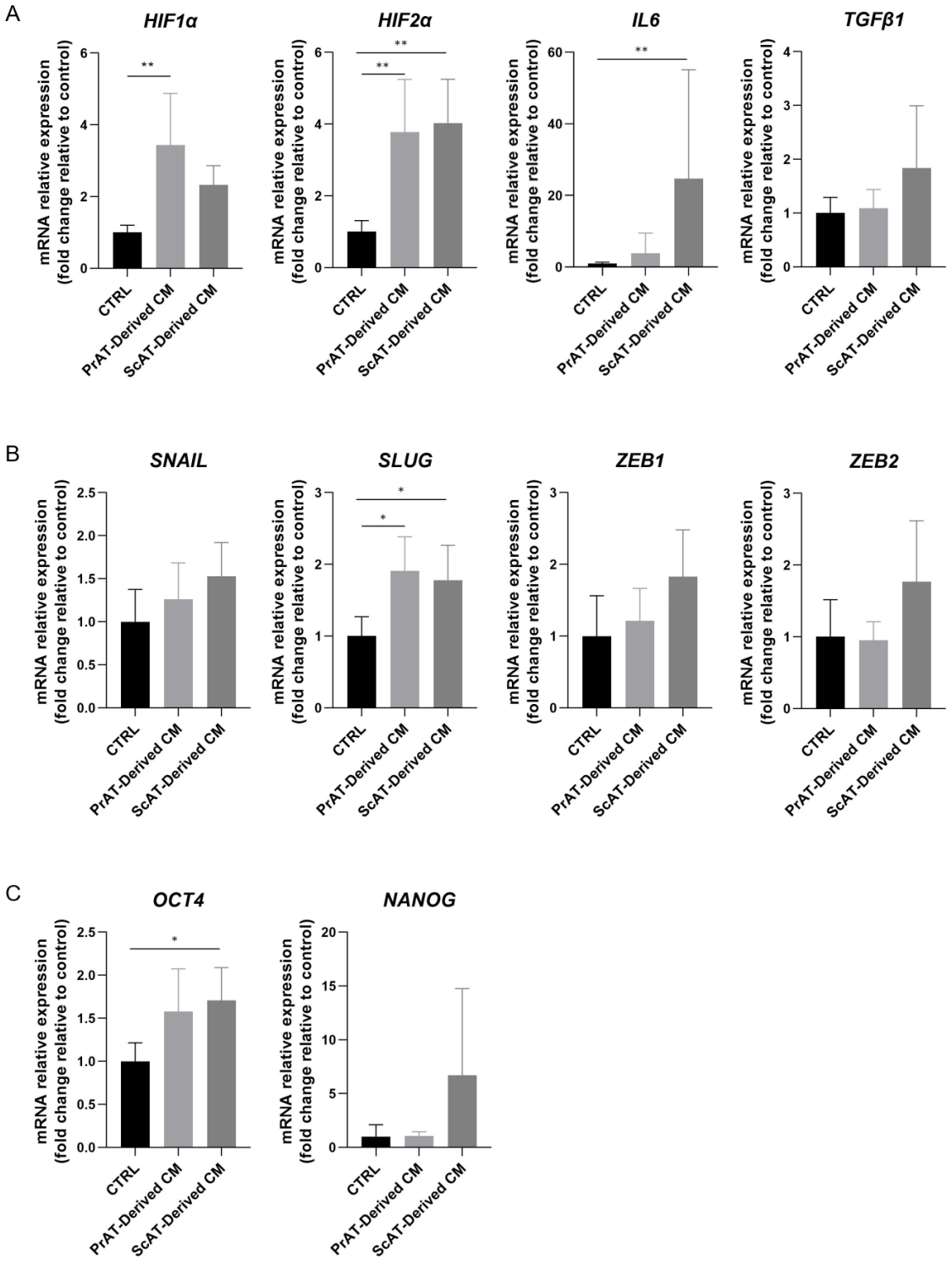


Figure 16 | **Adipose tissue-derived conditioned media induces alterations in mRNA relative expression of hypoxia-, inflammatory- (A), EMT- (B) and stem- (C) related genes in 786-O cells.** RNA extracted from 786-O cells cultured under the influence of perirenal and subcutaneous adipose tissue or cultured under normal conditions (CTRL) was reverse transcribed and relative mRNA levels were obtained by quantitative real-time PCR (n=5). Relative gene expression was normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) mRNA levels. Bars represent mean values and error bars represent standard deviation (SD). *p < 0.05 and **p<0.01 as determined by One-way ANOVA with Tukey's multiple comparisons test.

Increased levels of hypoxia and inflammatory associated genes could be found in cells cultured under the influence of both adipose depots' supernatants when compared to unstimulated cells (**Figure 16A**). In particular, 786-O cells cultured with PrAT-derived CM significantly increased the expression of hypoxia inducible factors (*HIF1α and HIF2α*). In turn, ScAT secreted factors mainly increased *HIF2α* and *IL6* expression in these cells (**Figure 16A**). No major differences were noticed in regard to *TGFβ1* relative expression.

When assessing the expression of major epithelial to mesenchymal transition transcription factors (EMT-TFs), an overall increase of the mRNA relative expression could be seen in 786-O cells cultured under the influence of both AT-derived CM when compared with control counterparts. Notably, perirenal and subcutaneous secreted factors significantly increased *SLUG* levels (**Figure 16B**).

Once EMT is described to induce cancer stemness in different models, the analysis of two stem-related genes in ccRCC was performed (**Figure 16C**). *OCT4* mRNA relative expression was increased in 786-O cells stimulated with soluble factors derived from perirenal and subcutaneous adipose tissue *ex vivo* culture when compared to control. However, *NANOG* only increased its levels whenever 786-O cells were cultured under the influence of ScAT-derived CM.

Altogether, PrAT and ScAT secreted factors influenced 786-O gene expression profile.

Adipose Tissue Soluble Factors Have No Effect on Caki-1 Cells Gene Expression

A similar approach to the one used in the previous chapter was used in Caki-1 cells. Caki-1 under the influence of AT-derived soluble factors revealed no significant differences in mRNA expression levels of tumour-promoting genes (**Figure 17**).

Figures 17A and **B** showed similar levels of mRNA expression for both genes involved in ccRCC developing process and EMT-TFs. However, even though a statistically significative difference could not be found, a slight increase in genes encoding stem-like markers in ccRCC could be observed (**Figure 17C**), suggesting a possible role for AT systemic factors in ccRCC stemness.

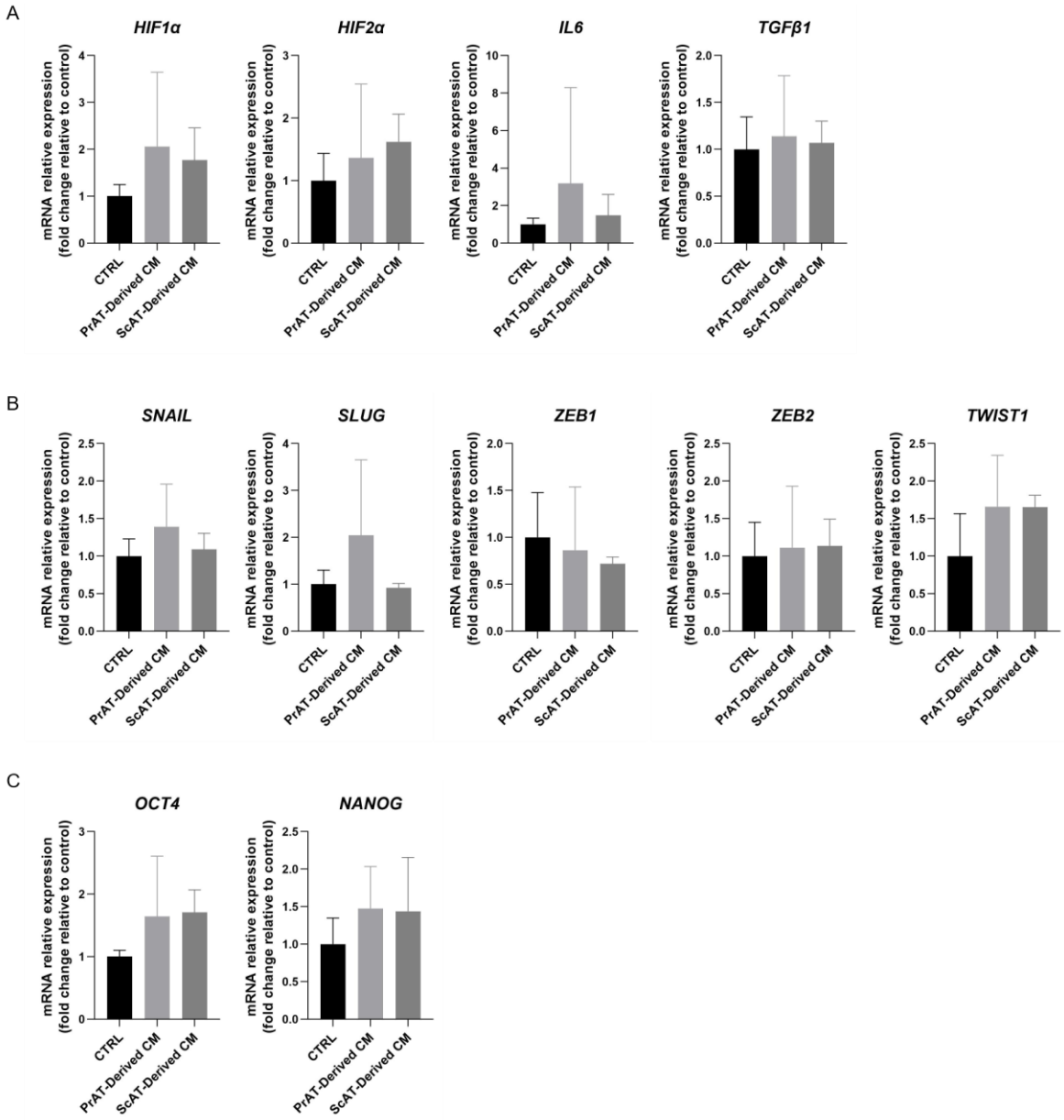


Figure 17 | **Adipose tissue-derived conditioned media does not appear to impact mRNA relative expression of tumour promoting- (A), EMT- (B) and stem- (C) related genes in Caki-1 cells**. RNA extracted from Caki-1 cells cultured under the influence of perirenal and subcutaneous adipose tissue or cultured under normal conditions (CTRL) was reverse transcribed and relative mRNA levels were obtained by quantitative real-time PCR (n=5). Relative gene expression was normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) mRNA levels. Bars represent mean values and error bars represent standard deviation (SD). Statistics determined by One-way ANOVA with Tukey's multiple comparisons test.

Adipose Tissue-Derived CM Inhibits Cell Proliferation of 786-O Cells

Following gene expression analysis, 786-O and Caki-1 cells cultured under the influence of PrAT or ScAT supernatants or under normal conditions were functionally characterized. Initially, the proliferating rates of both cell lines were determined resorting to the thymidine analog EdU (**Figure 18**). Both PrAT- and ScAT-derived CM impaired 786-O proliferation rates when compared to control (**Figure 18A** and **B**). In fact, 786-O actively proliferating cells were reduced in approximately 15% when cultured with both AT-derived CM in comparison to control (**Figure 18B**). In turn, no influence could be seen regarding the stimulation of Caki-1 with PrAT- and ScAT-derived CM (**Figure 18C** and **D**) in all three conditions.

Importantly, an overall effect of PrAT- and ScAT-derived CM in 786-O cells could be noticed, impairing its growth *in vitro*, underlying a role of these adipose depots to localized primary ccRCC tumours.

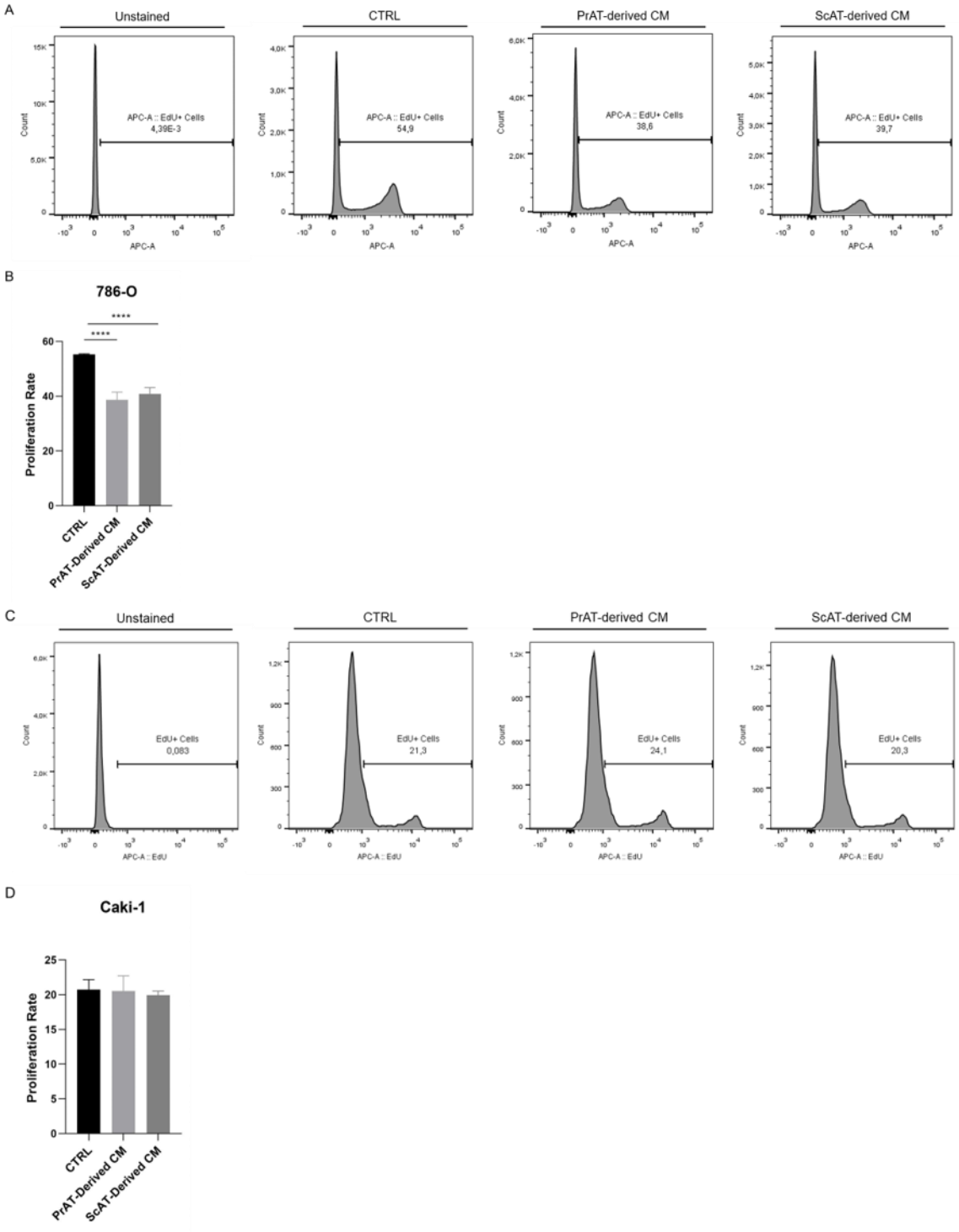


Figure 18 | **Proliferation rates of 786-O and Caki-1 cell lines.** Proliferative rates of 786-O and Caki-1 cells cultured under the influence of PrAT- or ScAT-derived CM or under normal conditions (CTRL) was assessed resorting to an EdU positive incorporation assay. EdU is a thymidine analog that is incorporated into newly synthesized DNA, allowing the measurement of cell proliferation. (A) Representative histograms of unstained and Edu^{+} cells in 786-O cells. (B) Actively proliferating 786-O cells cultured with and without adipose-derived CM (n=5). (C) Representative histograms of unstained and Edu^{+} cells in Caki-1 cells. (D) Actively proliferating Caki-1 cells cultured with and without adipose-derived CM (n=5). Bars represent mean values and error bars represent standard deviation (SD). ****$p < 0.0001$ as determined by One-way ANOVA with Tukey's multiple comparisons test.

Adipose Tissue Derived CM Promotes 786-O Apoptosis

Evasion of apoptosis is a well-established hallmark of cancer. In order to investigate the role of PrAT and ScAT-derived soluble factors on this mechanism, 786-O and Caki-1 cells cultured under the influence of perirenal or subcutaneous CM or under normal conditions were analysed by flow cytometry, following staining with annexin V and propidium iodide (**Figure 19**). This staining precedes the loss of membrane integrity and allows the distinction between live, apoptotic and necrotic cells.

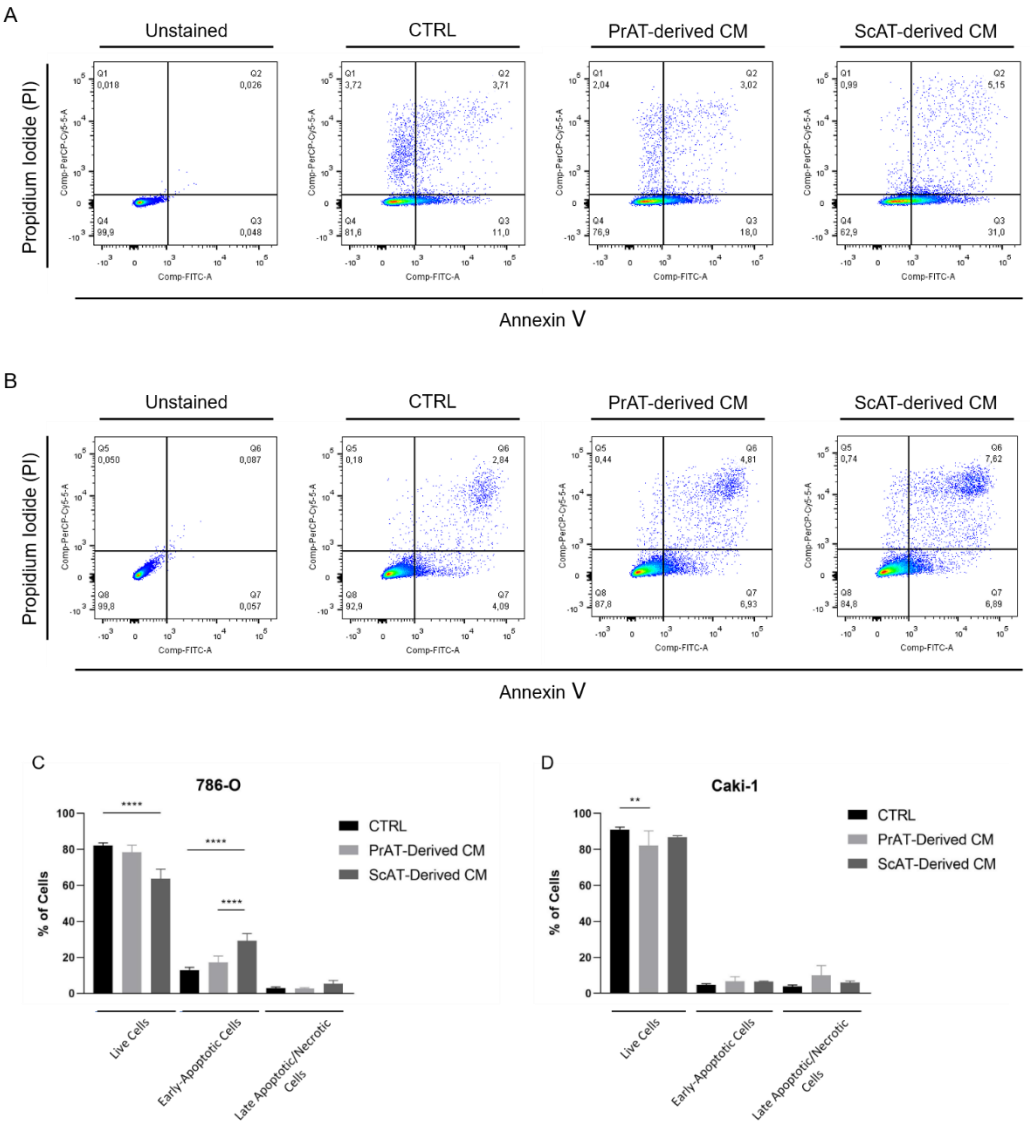


Figure 19 | **Effect of perirenal and subcutaneous adipose tissue-derived conditioned media on the cell apoptosis of 786-O (A) and Caki-1 cells (B)**. Apoptosis was measured as the incorporation of annexin V and propidium iodide (PI) in 786-O (A) or Caki-1 (B) cells. Cells cultured under the influence of PrAT- or ScAT-derived CM or under normal conditions (CTRL) were stained with annexin V and PI. Representative dot plots of live (annexin$^-$PI$^-$ cells), early-apoptotic (annexin$^+$PI$^-$) and late-apoptotic/necrotic (annexin$^+$PI$^+$) cells are depicted in (A) for 786-O cells (n=5) and (B) for Caki-1 cells (n=5). (C and D) Representative graph bars for the rate of live, early-apoptotic, and late-apoptotic cells in 786-O cells (C) and Caki-1 cells (D). **p < 0.01 and ***p < 0.0001 as determined by One-way ANOVA with Tukey's multiple comparisons test.

Soluble factors from PrAT *ex vivo* culture increased the percentage of both 786-O early apoptotic and late apoptotic/necrotic cells when compared to control (**Figure 19A**). This effect was even bigger when 786-O cells were cultured under the influence of ScAT-derived secreted mediators (**Figure 19A**). In fact, the subcutaneous adipose influence over 786-O cells surpassed the one exhibited by both PrAT and or control conditions (**Figure 19C**). Caki-1 cells decreased the number of live cells when stimulated with PrAT-derived CM when compared to control. However, no major differences could be seen between stimulated and control cells relatively to the number of cells that undergo apoptosis. (**Figure 19B** and **D**).

Overall, subcutaneous adipose tissue secreted factors appeared to promote 786-O cells apoptotic processes. This effect was less pronounced when cells were cultured under the influence of perirenal-derived CM. These data suggest that the subcutaneous depot can have a systemic effect on ccRCC primary tumours modulating apoptotic processes. Moreover, and even though PrAT did not possess such a conclusive effect in 786-O cells, the results also suggest that the adjacent perirenal adipose depot may play a role in the apoptotic behaviour of ccRCC primary tumours.

DISCUSSION

Altered microenvironment within a tissue is thought to provide permissive signals for tumour development. Importantly, adipocyte-secreted leptin was found to be upregulated in obese patients and different studies have suggested a role for leptin as a tumour growth promoter[184,185], by inducing STAT3 signalling[186], and consequently promoting proliferation and angiogenic differentiation of leptin receptor-expressing endothelial cells[187,188]. Contrarily, in the obese state, downregulated adiponectin secretion impairs its role on inhibiting tumour growth through the binding and sequestration of growth factors[172]. In addition, adiponectin reduced tumourigenesis in several *in vivo* studies[189,190] and its deficiency promoted tumour growth[191]. Furthermore, the production of pro-inflammatory adipokines, importantly, IL-6, has been shown to promote tumour growth in different types of cancer[192,193]. Adipocyte-derived IL-6 has been shown to induce an EMT phenotype in breast cancer cells via STAT3 signalling[194]. However, the antitumour effect of IL-6 blocking was only observed in high fat diet-induced mice, but not in controls, suggesting that the inflammatory state during obese conditions can influence the tumour outcome[195]. Additionally, adipocytes can modify the immune microenvironment by recruiting pro-inflammatory macrophages through the CCL2/IL-1β/CXCL12 axis, creating a permissive niche for cancer initiation[143]. All these findings support the interest on adipocytes and cancer crosstalk, which can be crucial for the prevention and treatment of obesity-associated cancers. Tumours developing within this favorable niche eventually progress towards aggressive, metastatic phenotypes[196]. Given the upsurge of the prevalence of obesity worldwide to pandemic levels, the disclosure of the mechanisms underlying the association between obesity and different types of cancer is urgently needed. In fact, a growing body of epidemiological data has made clear the association between obesity and increased cancer risk[106,107,197]. Particularly, compelling evidence linking obesity with renal cell carcinoma has been reported[106,198-200]. Adipocyte hypertrophic growth disrupts the dynamic role of the adipose tissue in energy homeostasis, consecutively leading to inflammation, modifications of adipokine signalling, lipid deregulation and shedding of mesenchymal-like cells from adipose depots to tumour niches. The plethora of abnormal processes that occur during obesity fosters cancer development and progression[164]. However, even though excess body adiposity is directly associated with increased risk for RCC development, obese patients at diagnosis experience longer survival than non-obese patients, a phenomenon designated “obesity paradox”[201]. To address this matter, a better understanding of the mechanistic processes that link adipose tissue to RCC progression is required.

This thesis sought to provide a study over the influence of different adipose depots on clear cell renal cell carcinoma (ccRCC), particularly the perirenal at the vicinity of RCC and subcutaneous adipose tissue, by addressing their role on ccRCC cell lines. Even though tumours are highly complex 3D structures, cell-line based research has contributed to a plethora of discoveries and, importantly, to the development of cancer therapies and effective drugs that were later on introduced into practice[202]. In line with this, two different ccRCC cell lines were used for the development of this work. To understand if differences between site-specific adipose depots and distinct ccRCC genetic profiles and tumour progression exist, 786-O and Caki-1 RCC lines were used. Whereas 786-O cells are defective in *VHL* expression and mimic ccRCC primary tumours, the Caki-1 RCC line is a model of distant metastatic ccRCC harboring wild-type *VHL*[203]. This allowed mimicking *in vitro* the influence of distant or tumour adjacent adipose depots in metastatic or localized ccRCC.

Importantly, a thorough characterization of the different adipose depots can contribute to a better understanding of the adipose tissue potential for cancer interactions. In this regard, both adipose depots were characterized with the employment of morphological, genetic and functional approaches. Preliminary results indicated that the perirenal adipose tissue (PrAT) presented decreased adipocyte size, as opposed to subcutaneous adipose tissue (ScAT). In fact, a reduction on the adipocyte area is consistent with the fact that adipocytes that exhibit a brown-like phenotype decrease size due to its lipolytic and thermogenic roles[204]. Additionally, gene expression profiling of the different adipose depots revealed a higher expression of thermogenic-related genes (including *UCP1*, *EVA1* and *TMEM26*) on PrAT when compared to ScAT. Importantly, even though no statistically significant difference could be found, the overall *UCP1* mRNA levels in PrAT were over 20-fold the expression of the same marker in ScAT. These data are in line with a number of reports that argue that the perirenal adipose tissue is a brown adipose depot[60,205-207]. Counterintuitively, even though PrAT displays higher relative expression levels of genes involved in brown phenotypic traits, SLC7A10 levels, a white adipocyte-specific cell surface protein, increased in the perirenal depot relatively to subcutaneous adipose tissue, as well. Defining adipose depots according to white, beige and brown composition is particularly difficult in humans since most tissues present heterogeneous populations of adipocytes[63]. In accordance, the relative gene expression levels observed for the perirenal depot might be the result of an intra-depot heterogeneity. In fact, a recent report portrayed the existence of dormant BAT within the perirenal depot that could be reactivated upon external stimuli. In concordance with the data presented here, the authors claim that similarly to subcutaneous WAT, dormant perirenal BAT was also characterized

by a unilocular morphology and identical gene expression profile in regard to ScAT[208]. Overall, here, we present evidences supporting that the perirenal adipocyte repertoire exhibit a BAT-like phenotype in contrast to subcutaneous WAT.

Following *ex vivo* culture of adipose tissue explants and respective conditioned media (CM) collection, a first approach to unravel the functional role of these tissues focused on the proteolytic activity that these depots possess. Matrix metalloproteinases (MMPs) are enzymes with proteolytic capabilities critical for the remodelling of the extracellular matrix (ECM) that have been associated with invasive and metastatic tumour behaviour. MMPs are secreted by both stroma and cancer cells and mediate several processes, including growth, apoptosis, migration and invasion of cancer cells, as well as regulation of tumour angiogenesis and immune tolerance[183,209,210]. Peritumoural adipose depots have already been described to promote invasive properties of tumour cells, directly by the production of multiple MMPs or through chemotactic agents[148,211]. The proteolytic activity of MMP2 and MMP9 was increased in the perirenal in opposite to subcutaneous depots, underlying a hypothetic effect of the tumour on the adjacent adipose tissue and remaining stroma to increase the levels of proteolytic enzymes to promote tumour invasion and/or metastasis. Further data is required to substantiate this premise.

At this point, the scope of this thesis shifted towards a better understanding of the influence that soluble factors secreted by two distinct anatomical fat depots had on ccRCC development and progression. Functional analyses of 786-O and Caki-1 RCC lines cultured under the influence of both PrAT- or ScAT-derived supernatants were conducted. These focused on the proliferative and apoptotic characteristics of these cells. In fact, the hind referred processes are established hallmarks of cancer[12]. 786-O cells displayed decreased proliferative rates and increased cell death when compared to control. Most studies report a protumourigenic role for the adipose tissue. Adipocyte-related cancer progression is thought to occur through proliferative and anti-apoptotic processes that promote malignancy. Concomitantly, the adipokine repertoire has a critical impact on cancer cell traits. In fact, adipocyte secreted factors, including leptin, IL-6, TNF-α and VEGF have angiogenic and proliferative effects over the growing tumour mass[113,196,212]. Furthermore, cancer-associated adipocytes undergo lipolysis that serves as a source of energy for cancer cells, promoting tumour growth[213]. In contrast, a tendency to decrease actively proliferating cells when 786-O RCC line is cultured under the influence of both analysed depots in comparison to unstimulated cells could be seen. Concordantly, these cells also displayed higher apoptotic rates than cells cultured under normal conditions. These results suggest a role for adipose tissue in tumour mass regression, giving some insight over the obesity paradox that is seen in RCC patients. Importantly, the inflammatory signals released from

the adipose tissue, that are exacerbated upon obesity, might be the underlying cause for the recruitment of immune populations, inducing the production of reactive oxygen species and consequent genotoxic stress[124,126]. To limit genomic instability, cells are equipped with DNA damage response elements and DNA repair proteins that contribute to the repair and tolerance of DNA lesions. Dysregulated repair mechanisms and the underlying genotoxic stress promote pathways of apoptotic and cell death, that are ultimately controlled by a threshold between pro-survival and pro-apoptotic factors[214-216]. Moreover, adipose-derived stem cells (ASCs) were shown to inhibit prostate cancer cell proliferation and induce apoptosis, in accordance to the data presented here[217]. These effects might be related to the action of adiponectin on proliferation and apoptotic processes, once this adipokine is reported to increase apoptotic signalling and reduce proliferation in cancer progression[218]. The combined evidence might suggest that adiponectin or the stromal compartment of the different adipose depots might constitute crucial players in cancer regression. However, the exact molecular mechanisms through which the adipose soluble factors contribute to these mechanisms are unknown, and further analysis is required.

Given the absence of VHL in 786-O cells, these cells already express high amounts of hypoxia inducible factors (HIF). However, upon exposure of these cells to AT-derived conditioned medium from both perirenal and subcutaneous depots, the mRNA levels of these transcription factors are further increased. Moreover, the gene expression levels of pro-inflammatory (*IL6*), EMT-associated (*SLUG*) and stem-related genes (*OCT4*) are increased in 786-O cells cultured under the influence of adipose depots supernatants. These data oppose to some extent the results obtained from the functional analysis performed on the same cells. This indicates that, even though 786-O cells are increasing its apoptotic rates and decreasing its proliferative capability, the surviving cells acquire a more aggressive phenotype somehow. Even though the hypothetic genotoxic stress decreases 786-O viability, the cells that are capable to survive seem to acquire a more aggressive phenotype, concomitant with the increased expression of both EMT-TFs and stemness-related markers. An emerging hypothesis relies on the possibility that the adipose tissue might increase the senescent cell burden in the developing tumour. Cell senescence denotes a durable form of cell-cycle arrest caused by multiple stress-related processes, including telomere dysfunction, genotoxic stress, inflammation and metabolic dysregulation[219]. Tumour cells that are induced into a state of senescence throughout chemotherapeutic or radiation approaches have been reported to recover self-renewal capacity and increase cancer aggressiveness[220]. In fact, a population of pseudo-dormant cells that evade the cytotoxic impact that adipose tissue-derived soluble factors might have on 786-O cells, might resurge with a more aggressive phenotype. Notably, these cells are

thought to increase the population of cancer stem cells, being the underlying cause for therapy resistance and disease recurrence[221]. Overall, both PrAT- and ScAT-derived supernatants increase the expression of different genes that influence tumour progression and development through a hypothetic selective process, whereby cells that survive the initial genotoxic effects of adipose tissue prosper within the established microenvironment and drive tumour growth, invasion and metastatic behaviour. Obviously, the conclusions drawn from this work relative to the influence of different adipose depots on ccRCC primary tumours remain to be proven and will be the focus of future work.

In regard to Caki-1 cells, the increased metastatic potential of these cell line, and the intrinsic phenotypic aggressiveness it displays, hampered the effects of AT-derived CM. Moreover, it is somehow expected that adjacent tumour adipose tissue might have a direct influence on cancer cells phenotypic changes, particularly, when the evading tumour mass reaches the adipose organ. However, in 786-O cells an effect of nearby and distant adipose tissue soluble factors could be seen implying paracrine and systemic influences over the localized ccRCC cell line. In turn, Caki-1 cells, which derive from a metastatic site, do not respond to both AT secreted factors influence. Overall, it seems that adipose tissue might promote tumour aggressiveness in a site-specific manner.

It is important to notice that, several methodological approaches and/or sample collection deficits might influence the different effects seen in both cell lines, and importantly, the absence of effects detected in Caki-1 cells cultured under the influence of AT supernatants. Incubation periods, seeding densities and the stratification of patients according to clinical and pathological data might be critical to increase the importance of this study for the clinical practice. In fact, the limiting number of samples collected (due to temporal restriction) is a critical factor that hindered this work. In conclusion, this study sets some evidence over the influence of different adipose depots on renal cell carcinoma progression and aims at providing data to conduct future insights in the adipose tissue/RCC interface, including the development of new therapeutic approaches that target both adjacent and/or distant adipose tissue as well as the tumour itself.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Tumours largely depend on their tumour microenvironment for sustained growth, invasion and metastasis. Targeting the tumour microenvironment non-transformed cells represents an attractive therapeutic strategy. Adipocytes have been a neglected component of this landscape. However, in recent years, increased interest has been laid upon the role of this cell type to tumour initiation and progression. In fact, several studies have disclosed a robust association between obesity and increased risk to develop several types of cancer, making the concept of obesity indissociable from cancer. Importantly, renal cell carcinoma is among the cancers that are highly associated with obesity. Disclosing the role of adipose tissue towards RCC progression is thus of crucial importance. Even though multiple factors could explain the increased incidence of RCC in obesity, including dysfunctional adipokine signalling, increased leptin and lipids, decreased adiponectin and inflammatory signalling, the mechanistic processes behind these are largely unknown. The work herein developed reflects the influence that both the perirenal and the subcutaneous adipose depots might have for RCC carcinogenic processes and sets its basis on the development of future work that aims to disclose the mechanistic insights behind these processes, and the discovery and development of novel therapeutics.

Future work following on this thesis will seek to unveil the precise mechanisms through which the adipose depot influences renal cell carcinoma, with a focus on immunometabolic and cellular energetics processes, as well as in the related signalling pathways. In line with this, three main objectives should be pursued:

1. To unravel the role of secreted factors to RCC growth. Establishing the differences of site-specific adipose adipokine repertoire, emphasizing the immunomodulatory role of these secreted factors.
2. To unveil the distinct immune populations that are part of the adipose landscape of both peritumoural and distant adipose depots that may ultimately contribute towards disease.
3. To establish the influence of the metabolic symbiosis phenomenon on RCC and unravel the role of lipid metabolism to tumour cell growth.

A better understanding of these mechanisms and concurrent influence on cancer progression should pave the way to increase the knowledge over the influence of the

adipose tissue over RCC and to develop new therapeutic approaches that target not only cancer cells, but the adipose landscape.

REFERENCES

1 Capitanio, U. *et al.* Epidemiology of Renal Cell Carcinoma. *Eur Urol* **75**, 74-84, doi:10.1016/j.eururo.2018.08.036 (2019).
2 Capitanio, U. & Montorsi, F. Renal cancer. *Lancet* **387**, 894-906, doi:10.1016/S0140-6736(15)00046-X (2016).
3 Hsieh, J. J. *et al.* Renal cell carcinoma. *Nat Rev Dis Primers* **3**, 17009, doi:10.1038/nrdp.2017.9 (2017).
4 Bhatt, J. R. & Finelli, A. Landmarks in the diagnosis and treatment of renal cell carcinoma. *Nat Rev Urol* **11**, 517-525, doi:10.1038/nrurol.2014.194 (2014).
5 Cancer Genome Atlas Research, N. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* **499**, 43-49, doi:10.1038/nature12222 (2013).
6 Sato, Y. *et al.* Integrated molecular analysis of clear-cell renal cell carcinoma. *Nat Genet* **45**, 860-867, doi:10.1038/ng.2699 (2013).
7 Hakimi, A. A. *et al.* An Integrated Metabolic Atlas of Clear Cell Renal Cell Carcinoma. *Cancer Cell* **29**, 104-116, doi:10.1016/j.ccell.2015.12.004 (2016).
8 Gerlinger, M. *et al.* Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. *Nat Genet* **46**, 225-233, doi:10.1038/ng.2891 (2014).
9 Gerlinger, M. *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* **366**, 883-892, doi:10.1056/NEJMoa1113205 (2012).
10 Rini, B. I., Campbell, S. C. & Escudier, B. Renal cell carcinoma. *Lancet* **373**, 1119-1132, doi:10.1016/S0140-6736(09)60229-4 (2009).
11 Porta, C. *et al.* The adjuvant treatment of kidney cancer: a multidisciplinary outlook. *Nat Rev Nephrol* **15**, 423-433, doi:10.1038/s41581-019-0131-x (2019).
12 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).
13 Linehan, W. M. & Ricketts, C. J. The Cancer Genome Atlas of renal cell carcinoma: findings and clinical implications. *Nat Rev Urol* **16**, 539-552, doi:10.1038/s41585-019-0211-5 (2019).
14 Mitchell, T. J. *et al.* Timing the Landmark Events in the Evolution of Clear Cell Renal Cell Cancer: TRACERx Renal. *Cell* **173**, 611-623 e617, doi:10.1016/j.cell.2018.02.020 (2018).
15 Linehan, W. M., Srinivasan, R. & Schmidt, L. S. The genetic basis of kidney cancer: a metabolic disease. *Nat Rev Urol* **7**, 277-285, doi:10.1038/nrurol.2010.47 (2010).
16 Sanchez, D. J. & Simon, M. C. Transcriptional control of kidney cancer. *Science* **361**, 226-227, doi:10.1126/science.aau4385 (2018).
17 Joosten, S. C. *et al.* Epigenetics in renal cell cancer: mechanisms and clinical applications. *Nat Rev Urol* **15**, 430-451, doi:10.1038/s41585-018-0023-z (2018).
18 Varela, I. *et al.* Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature* **469**, 539-542, doi:10.1038/nature09639 (2011).
19 Morris, M. R. & Latif, F. The epigenetic landscape of renal cancer. *Nat Rev Nephrol* **13**, 47-60, doi:10.1038/nrneph.2016.168 (2017).
20 Espana-Agusti, J., Warren, A., Chew, S. K., Adams, D. J. & Matakidou, A. Loss of PBRM1 rescues VHL dependent replication stress to promote renal carcinogenesis. *Nat Commun* **8**, 2026, doi:10.1038/s41467-017-02245-1 (2017).
21 Nargund, A. M. *et al.* The SWI/SNF Protein PBRM1 Restrains VHL-Loss-Driven Clear Cell Renal Cell Carcinoma. *Cell Rep* **18**, 2893-2906, doi:10.1016/j.celrep.2017.02.074 (2017).
22 Pena-Llopis, S. *et al.* BAP1 loss defines a new class of renal cell carcinoma. *Nat Genet* **44**, 751-759, doi:10.1038/ng.2323 (2012).

23 Nishikawa, H. *et al.* BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity. *Cancer Res* **69**, 111-119, doi:10.1158/0008-5472.CAN-08-3355 (2009).
24 Hakimi, A. A. *et al.* Adverse outcomes in clear cell renal cell carcinoma with mutations of 3p21 epigenetic regulators BAP1 and SETD2: a report by MSKCC and the KIRC TCGA research network. *Clin Cancer Res* **19**, 3259-3267, doi:10.1158/1078-0432.CCR-12-3886 (2013).
25 Zhang, Y. *et al.* BAP1 links metabolic regulation of ferroptosis to tumour suppression. *Nat Cell Biol* **20**, 1181-1192, doi:10.1038/s41556-018-0178-0 (2018).
26 Carvalho, S. *et al.* SETD2 is required for DNA double-strand break repair and activation of the p53-mediated checkpoint. *Elife* **3**, e02482, doi:10.7554/eLife.02482 (2014).
27 Kanu, N. *et al.* SETD2 loss-of-function promotes renal cancer branched evolution through replication stress and impaired DNA repair. *Oncogene* **34**, 5699-5708, doi:10.1038/onc.2015.24 (2015).
28 Hakimi, A. A., Pham, C. G. & Hsieh, J. J. A clear picture of renal cell carcinoma. *Nat Genet* **45**, 849-850, doi:10.1038/ng.2708 (2013).
29 Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat Rev Cancer* **9**, 239-252, doi:10.1038/nrc2618 (2009).
30 Turley, S. J., Cremasco, V. & Astarita, J. L. Immunological hallmarks of stromal cells in the tumour microenvironment. *Nat Rev Immunol* **15**, 669-682, doi:10.1038/nri3902 (2015).
31 O'Donnell, J. S., Teng, M. W. L. & Smyth, M. J. Cancer immunoediting and resistance to T cell-based immunotherapy. *Nat Rev Clin Oncol* **16**, 151-167, doi:10.1038/s41571-018-0142-8 (2019).
32 Chevrier, S. *et al.* An Immune Atlas of Clear Cell Renal Cell Carcinoma. *Cell* **169**, 736-749 e718, doi:10.1016/j.cell.2017.04.016 (2017).
33 Efremova, M. *et al.* Targeting immune checkpoints potentiates immunoediting and changes the dynamics of tumor evolution. *Nat Commun* **9**, 32, doi:10.1038/s41467-017-02424-0 (2018).
34 Huang, Y. *et al.* Clonal architectures predict clinical outcome in clear cell renal cell carcinoma. *Nat Commun* **10**, 1245, doi:10.1038/s41467-019-09241-7 (2019).
35 Senbabaoglu, Y. *et al.* Tumor immune microenvironment characterization in clear cell renal cell carcinoma identifies prognostic and immunotherapeutically relevant messenger RNA signatures. *Genome Biol* **17**, 231, doi:10.1186/s13059-016-1092-z (2016).
36 Courtney, K. D. *et al.* Isotope Tracing of Human Clear Cell Renal Cell Carcinomas Demonstrates Suppressed Glucose Oxidation In Vivo. *Cell Metab* **28**, 793-800 e792, doi:10.1016/j.cmet.2018.07.020 (2018).
37 Hassan, M., Latif, N. & Yacoub, M. Adipose tissue: friend or foe? *Nat Rev Cardiol* **9**, 689-702, doi:10.1038/nrcardio.2012.148 (2012).
38 Bornfeldt, K. E. & Tabas, I. Insulin resistance, hyperglycemia, and atherosclerosis. *Cell Metab* **14**, 575-585, doi:10.1016/j.cmet.2011.07.015 (2011).
39 Harms, M. & Seale, P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* **19**, 1252-1263, doi:10.1038/nm.3361 (2013).
40 Klil-Drori, A. J., Azoulay, L. & Pollak, M. N. Cancer, obesity, diabetes, and antidiabetic drugs: is the fog clearing? *Nat Rev Clin Oncol* **14**, 85-99, doi:10.1038/nrclinonc.2016.120 (2017).
41 Cristancho, A. G. & Lazar, M. A. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol* **12**, 722-734, doi:10.1038/nrm3198 (2011).
42 Sanchez-Gurmaches, J., Hung, C. M. & Guertin, D. A. Emerging Complexities in Adipocyte Origins and Identity. *Trends Cell Biol* **26**, 313-326, doi:10.1016/j.tcb.2016.01.004 (2016).
43 Ikeda, K., Maretich, P. & Kajimura, S. The Common and Distinct Features of Brown and Beige Adipocytes. *Trends Endocrinol Metab* **29**, 191-200, doi:10.1016/j.tem.2018.01.001 (2018).

44 Wang, W. & Seale, P. Control of brown and beige fat development. *Nat Rev Mol Cell Biol* **17**, 691-702, doi:10.1038/nrm.2016.96 (2016).
45 Cypess, A. M. *et al.* Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* **19**, 635-639, doi:10.1038/nm.3112 (2013).
46 Deng, Y. & Scherer, P. E. Adipokines as novel biomarkers and regulators of the metabolic syndrome. *Ann N Y Acad Sci* **1212**, E1-E19, doi:10.1111/j.1749-6632.2010.05875.x (2010).
47 Lidell, M. E. & Enerback, S. Brown adipose tissue--a new role in humans? *Nat Rev Endocrinol* **6**, 319-325, doi:10.1038/nrendo.2010.64 (2010).
48 Jeffery, E. *et al.* The Adipose Tissue Microenvironment Regulates Depot-Specific Adipogenesis in Obesity. *Cell Metab* **24**, 142-150, doi:10.1016/j.cmet.2016.05.012 (2016).
49 Peirce, V., Carobbio, S. & Vidal-Puig, A. The different shades of fat. *Nature* **510**, 76-83, doi:10.1038/nature13477 (2014).
50 Cannon, B. & Nedergaard, J. Brown adipose tissue: function and physiological significance. *Physiol Rev* **84**, 277-359, doi:10.1152/physrev.00015.2003 (2004).
51 Shabalina, I. G. *et al.* UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. *Cell Rep* **5**, 1196-1203, doi:10.1016/j.celrep.2013.10.044 (2013).
52 Fedorenko, A., Lishko, P. V. & Kirichok, Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* **151**, 400-413, doi:10.1016/j.cell.2012.09.010 (2012).
53 Cypess, A. M. *et al.* Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. *Cell Metab* **21**, 33-38, doi:10.1016/j.cmet.2014.12.009 (2015).
54 Orava, J. *et al.* Different metabolic responses of human brown adipose tissue to activation by cold and insulin. *Cell Metab* **14**, 272-279, doi:10.1016/j.cmet.2011.06.012 (2011).
55 Cypess, A. M. *et al.* Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* **360**, 1509-1517, doi:10.1056/NEJMoa0810780 (2009).
56 Virtanen, K. A. *et al.* Functional brown adipose tissue in healthy adults. *N Engl J Med* **360**, 1518-1525, doi:10.1056/NEJMoa0808949 (2009).
57 van Marken Lichtenbelt, W. D. *et al.* Cold-activated brown adipose tissue in healthy men. *N Engl J Med* **360**, 1500-1508, doi:10.1056/NEJMoa0808718 (2009).
58 Cao, Y. Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. *Nat Rev Drug Discov* **9**, 107-115, doi:10.1038/nrd3055 (2010).
59 Chouchani, E. T. & Kajimura, S. Metabolic adaptation and maladaptation in adipose tissue. *Nat Metab*, 189-200, doi:10.1038/s42255-018-0021-8 (2019).
60 Lidell, M. E. *et al.* Evidence for two types of brown adipose tissue in humans. *Nat Med* **19**, 631-634, doi:10.1038/nm.3017 (2013).
61 Wu, J. *et al.* Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* **150**, 366-376, doi:10.1016/j.cell.2012.05.016 (2012).
62 Gesta, S. *et al.* Evidence for a role of developmental genes in the origin of obesity and body fat distribution. *Proc Natl Acad Sci U S A* **103**, 6676-6681, doi:10.1073/pnas.0601752103 (2006).
63 Ussar, S. *et al.* ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. *Sci Transl Med* **6**, 247ra103, doi:10.1126/scitranslmed.3008490 (2014).
64 Berry, R. & Rodeheffer, M. S. Characterization of the adipocyte cellular lineage in vivo. *Nat Cell Biol* **15**, 302-308, doi:10.1038/ncb2696 (2013).
65 Ghaben, A. L. & Scherer, P. E. Adipogenesis and metabolic health. *Nat Rev Mol Cell Biol* **20**, 242-258, doi:10.1038/s41580-018-0093-z (2019).
66 Tang, W. *et al.* White fat progenitor cells reside in the adipose vasculature. *Science* **322**, 583-586, doi:10.1126/science.1156232 (2008).
67 Quach, J. M. *et al.* Zinc finger protein 467 is a novel regulator of osteoblast and adipocyte commitment. *J Biol Chem* **286**, 4186-4198, doi:10.1074/jbc.M110.178251 (2011).

68 Gupta, R. K. *et al.* Transcriptional control of preadipocyte determination by Zfp423. *Nature* **464**, 619-623, doi:10.1038/nature08816 (2010).
69 Huang, H. *et al.* BMP signaling pathway is required for commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. *Proc Natl Acad Sci U S A* **106**, 12670-12675, doi:10.1073/pnas.0906266106 (2009).
70 Timmons, J. A. *et al.* Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A* **104**, 4401-4406, doi:10.1073/pnas.0610615104 (2007).
71 Sidossis, L. & Kajimura, S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest* **125**, 478-486, doi:10.1172/JCI78362 (2015).
72 Lepper, C. & Fan, C. M. Inducible lineage tracing of Pax7-descendant cells reveals embryonic origin of adult satellite cells. *Genesis* **48**, 424-436, doi:10.1002/dvg.20630 (2010).
73 Seale, P. *et al.* PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454**, 961-967, doi:10.1038/nature07182 (2008).
74 Ohno, H., Shinoda, K., Ohyama, K., Sharp, L. Z. & Kajimura, S. EHMT1 controls brown adipose cell fate and thermogenesis through the PRDM16 complex. *Nature* **504**, 163-167, doi:10.1038/nature12652 (2013).
75 Schulz, T. J. *et al.* Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc Natl Acad Sci U S A* **108**, 143-148, doi:10.1073/pnas.1010929108 (2011).
76 Wang, W. *et al.* Ebf2 is a selective marker of brown and beige adipogenic precursor cells. *Proc Natl Acad Sci U S A* **111**, 14466-14471, doi:10.1073/pnas.1412685111 (2014).
77 Shao, M. *et al.* Zfp423 Maintains White Adipocyte Identity through Suppression of the Beige Cell Thermogenic Gene Program. *Cell Metab* **23**, 1167-1184, doi:10.1016/j.cmet.2016.04.023 (2016).
78 Pope, B. D., Warren, C. R., Parker, K. K. & Cowan, C. A. Microenvironmental Control of Adipocyte Fate and Function. *Trends Cell Biol* **26**, 745-755, doi:10.1016/j.tcb.2016.05.005 (2016).
79 Cao, Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest* **117**, 2362-2368, doi:10.1172/JCI32239 (2007).
80 Cao, Y. Angiogenesis and vascular functions in modulation of obesity, adipose metabolism, and insulin sensitivity. *Cell Metab* **18**, 478-489, doi:10.1016/j.cmet.2013.08.008 (2013).
81 Gealekman, O. *et al.* Enhanced angiogenesis in obesity and in response to PPARgamma activators through adipocyte VEGF and ANGPTL4 production. *Am J Physiol Endocrinol Metab* **295**, E1056-1064, doi:10.1152/ajpendo.90345.2008 (2008).
82 Xue, Y. *et al.* Hypoxia-independent angiogenesis in adipose tissues during cold acclimation. *Cell Metab* **9**, 99-109, doi:10.1016/j.cmet.2008.11.009 (2009).
83 Friedman, J. M. & Halaas, J. L. Leptin and the regulation of body weight in mammals. *Nature* **395**, 763-770, doi:10.1038/27376 (1998).
84 Villarroya, F., Gavalda-Navarro, A., Peyrou, M., Villarroya, J. & Giralt, M. The Lives and Times of Brown Adipokines. *Trends Endocrinol Metab* **28**, 855-867, doi:10.1016/j.tem.2017.10.005 (2017).
85 Zeng, W. *et al.* Sympathetic neuro-adipose connections mediate leptin-driven lipolysis. *Cell* **163**, 84-94, doi:10.1016/j.cell.2015.08.055 (2015).
86 Wu, D. *et al.* Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* **332**, 243-247, doi:10.1126/science.1201475 (2011).
87 Qiu, Y. *et al.* Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* **157**, 1292-1308, doi:10.1016/j.cell.2014.03.066 (2014).

88 Nussbaum, J. C. *et al.* Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* **502**, 245-248, doi:10.1038/nature12526 (2013).
89 Cipolletta, D. *et al.* PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* **486**, 549-553, doi:10.1038/nature11132 (2012).
90 Feuerer, M. *et al.* Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A* **107**, 5919-5924, doi:10.1073/pnas.1002006107 (2010).
91 Medrikova, D. *et al.* Brown adipose tissue harbors a distinct sub-population of regulatory T cells. *PLoS One* **10**, e0118534, doi:10.1371/journal.pone.0118534 (2015).
92 Han, S. J. *et al.* White Adipose Tissue Is a Reservoir for Memory T Cells and Promotes Protective Memory Responses to Infection. *Immunity* **47**, 1154-1168 e1156, doi:10.1016/j.immuni.2017.11.009 (2017).
93 Weisberg, S. P. *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* **112**, 1796-1808, doi:10.1172/JCI19246 (2003).
94 Lumeng, C. N., Bodzin, J. L. & Saltiel, A. R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**, 175-184, doi:10.1172/JCI29881 (2007).
95 Zhu, Q. & Scherer, P. E. Immunologic and endocrine functions of adipose tissue: implications for kidney disease. *Nat Rev Nephrol* **14**, 105-120, doi:10.1038/nrneph.2017.157 (2018).
96 Bluher, M. & Mantzoros, C. S. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. *Metabolism* **64**, 131-145, doi:10.1016/j.metabol.2014.10.016 (2015).
97 Fasshauer, M. & Bluher, M. Adipokines in health and disease. *Trends Pharmacol Sci* **36**, 461-470, doi:10.1016/j.tips.2015.04.014 (2015).
98 Wang, G. X., Zhao, X. Y. & Lin, J. D. The brown fat secretome: metabolic functions beyond thermogenesis. *Trends Endocrinol Metab* **26**, 231-237, doi:10.1016/j.tem.2015.03.002 (2015).
99 Zhang, Y. *et al.* Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432, doi:10.1038/372425a0 (1994).
100 Farooqi, I. S. & O'Rahilly, S. 20 years of leptin: human disorders of leptin action. *J Endocrinol* **223**, T63-70, doi:10.1530/JOE-14-0480 (2014).
101 Friedman, J. The long road to leptin. *J Clin Invest* **126**, 4727-4734, doi:10.1172/JCI91578 (2016).
102 Scheja, L. & Heeren, J. The endocrine function of adipose tissues in health and cardiometabolic disease. *Nat Rev Endocrinol* **15**, 507-524, doi:10.1038/s41574-019-0230-6 (2019).
103 Calle, E. E., Rodriguez, C., Walker-Thurmond, K. & Thun, M. J. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* **348**, 1625-1638, doi:10.1056/NEJMoa021423 (2003).
104 O'Sullivan, J., Lysaght, J., Donohoe, C. L. & Reynolds, J. V. Obesity and gastrointestinal cancer: the interrelationship of adipose and tumour microenvironments. *Nat Rev Gastroenterol Hepatol* **15**, 699-714, doi:10.1038/s41575-018-0069-7 (2018).
105 Arnold, M. *et al.* Obesity and cancer: An update of the global impact. *Cancer Epidemiol* **41**, 8-15, doi:10.1016/j.canep.2016.01.003 (2016).
106 Renehan, A. G., Tyson, M., Egger, M., Heller, R. F. & Zwahlen, M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* **371**, 569-578, doi:10.1016/S0140-6736(08)60269-X (2008).
107 Bhaskaran, K. *et al.* Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5.24 million UK adults. *Lancet* **384**, 755-765, doi:10.1016/S0140-6736(14)60892-8 (2014).
108 Schauer, D. P. *et al.* Association Between Weight Loss and the Risk of Cancer after Bariatric Surgery. *Obesity (Silver Spring)* **25 Suppl 2**, S52-S57, doi:10.1002/oby.22002 (2017).

109 Allott, E. H., Masko, E. M. & Freedland, S. J. Obesity and prostate cancer: weighing the evidence. *Eur Urol* **63**, 800-809, doi:10.1016/j.eururo.2012.11.013 (2013).
110 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70, doi:10.1016/s0092-8674(00)81683-9 (2000).
111 Nieman, K. M., Romero, I. L., Van Houten, B. & Lengyel, E. Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochim Biophys Acta* **1831**, 1533-1541, doi:10.1016/j.bbalip.2013.02.010 (2013).
112 Park, J., Morley, T. S., Kim, M., Clegg, D. J. & Scherer, P. E. Obesity and cancer--mechanisms underlying tumour progression and recurrence. *Nat Rev Endocrinol* **10**, 455-465, doi:10.1038/nrendo.2014.94 (2014).
113 Lengyel, E., Makowski, L., DiGiovanni, J. & Kolonin, M. G. Cancer as a Matter of Fat: The Crosstalk between Adipose Tissue and Tumors. *Trends Cancer* **4**, 374-384, doi:10.1016/j.trecan.2018.03.004 (2018).
114 Wilson, W. R. & Hay, M. P. Targeting hypoxia in cancer therapy. *Nat Rev Cancer* **11**, 393-410, doi:10.1038/nrc3064 (2011).
115 Halberg, N. *et al.* Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol* **29**, 4467-4483, doi:10.1128/MCB.00192-09 (2009).
116 Sun, K., Tordjman, J., Clement, K. & Scherer, P. E. Fibrosis and adipose tissue dysfunction. *Cell Metab* **18**, 470-477, doi:10.1016/j.cmet.2013.06.016 (2013).
117 Park, J. & Scherer, P. E. Adipocyte-derived endotrophin promotes malignant tumor progression. *J Clin Invest* **122**, 4243-4256, doi:10.1172/JCI63930 (2012).
118 Sun, K. *et al.* Endotrophin triggers adipose tissue fibrosis and metabolic dysfunction. *Nat Commun* **5**, 3485, doi:10.1038/ncomms4485 (2014).
119 Vegiopoulos, A., Rohm, M. & Herzig, S. Adipose tissue: between the extremes. *EMBO J* **36**, 1999-2017, doi:10.15252/embj.201696206 (2017).
120 Elinav, E. *et al.* Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nat Rev Cancer* **13**, 759-771, doi:10.1038/nrc3611 (2013).
121 Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **454**, 428-435, doi:10.1038/nature07201 (2008).
122 Johnson, A. R., Milner, J. J. & Makowski, L. The inflammation highway: metabolism accelerates inflammatory traffic in obesity. *Immunol Rev* **249**, 218-238, doi:10.1111/j.1600-065X.2012.01151.x (2012).
123 Seo, B. R. *et al.* Obesity-dependent changes in interstitial ECM mechanics promote breast tumorigenesis. *Sci Transl Med* **7**, 301ra130, doi:10.1126/scitranslmed.3010467 (2015).
124 Kryston, T. B., Georgiev, A. B., Pissis, P. & Georgakilas, A. G. Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat Res* **711**, 193-201, doi:10.1016/j.mrfmmm.2010.12.016 (2011).
125 Luperini, B. C. *et al.* Gene polymorphisms and increased DNA damage in morbidly obese women. *Mutat Res* **776**, 111-117, doi:10.1016/j.mrfmmm.2015.01.004 (2015).
126 Azzara, A., Pirillo, C., Giovannini, C., Federico, G. & Scarpato, R. Different repair kinetic of DSBs induced by mitomycin C in peripheral lymphocytes of obese and normal weight adolescents. *Mutat Res* **789**, 9-14, doi:10.1016/j.mrfmmm.2016.05.001 (2016).
127 Olson, O. C., Quail, D. F. & Joyce, J. A. Obesity and the tumor microenvironment. *Science* **358**, 1130-1131, doi:10.1126/science.aao5801 (2017).
128 Santander, A. M. *et al.* Paracrine Interactions between Adipocytes and Tumor Cells Recruit and Modify Macrophages to the Mammary Tumor Microenvironment: The Role of Obesity and Inflammation in Breast Adipose Tissue. *Cancers (Basel)* **7**, 143-178, doi:10.3390/cancers7010143 (2015).
129 Sun, K., Kusminski, C. M. & Scherer, P. E. Adipose tissue remodeling and obesity. *J Clin Invest* **121**, 2094-2101, doi:10.1172/JCI45887 (2011).
130 Mayi, T. H. *et al.* Human adipose tissue macrophages display activation of cancer-related pathways. *J Biol Chem* **287**, 21904-21913, doi:10.1074/jbc.M111.315200 (2012).

131 Iyengar, N. M. *et al.* Metabolic Obesity, Adipose Inflammation and Elevated Breast Aromatase in Women with Normal Body Mass Index. *Cancer Prev Res (Phila)* **10**, 235-243, doi:10.1158/1940-6207.CAPR-16-0314 (2017).
132 Sun, X. *et al.* Normal breast tissue of obese women is enriched for macrophage markers and macrophage-associated gene expression. *Breast Cancer Res Treat* **131**, 1003-1012, doi:10.1007/s10549-011-1789-3 (2012).
133 Campbell, M. J. *et al.* Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast Cancer Res Treat* **128**, 703-711, doi:10.1007/s10549-010-1154-y (2011).
134 Lysaght, J. *et al.* Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue. *Cancer Lett* **312**, 62-72, doi:10.1016/j.canlet.2011.07.034 (2011).
135 Nishimura, S. *et al.* CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med* **15**, 914-920, doi:10.1038/nm.1964 (2009).
136 Noman, M. Z. *et al.* PD-L1 is a novel direct target of HIF-1alpha, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *J Exp Med* **211**, 781-790, doi:10.1084/jem.20131916 (2014).
137 Shirakawa, K. *et al.* Obesity accelerates T cell senescence in murine visceral adipose tissue. *J Clin Invest* **126**, 4626-4639, doi:10.1172/JCI88606 (2016).
138 Lynch, L. *et al.* Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. *Eur J Immunol* **39**, 1893-1901, doi:10.1002/eji.200939349 (2009).
139 Conroy, M. J. *et al.* The microenvironment of visceral adipose tissue and liver alter natural killer cell viability and function. *J Leukoc Biol* **100**, 1435-1442, doi:10.1189/jlb.5AB1115-493RR (2016).
140 Karnoub, A. E. *et al.* Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**, 557-563, doi:10.1038/nature06188 (2007).
141 Bhowmick, N. A., Neilson, E. G. & Moses, H. L. Stromal fibroblasts in cancer initiation and progression. *Nature* **432**, 332-337, doi:10.1038/nature03096 (2004).
142 Zhang, T. *et al.* CXCL1 mediates obesity-associated adipose stromal cell trafficking and function in the tumour microenvironment. *Nat Commun* **7**, 11674, doi:10.1038/ncomms11674 (2016).
143 Arendt, L. M. *et al.* Obesity promotes breast cancer by CCL2-mediated macrophage recruitment and angiogenesis. *Cancer Res* **73**, 6080-6093, doi:10.1158/0008-5472.CAN-13-0926 (2013).
144 Nowicka, A. *et al.* Human omental-derived adipose stem cells increase ovarian cancer proliferation, migration, and chemoresistance. *PLoS One* **8**, e81859, doi:10.1371/journal.pone.0081859 (2013).
145 Orecchioni, S. *et al.* Complementary populations of human adipose CD34+ progenitor cells promote growth, angiogenesis, and metastasis of breast cancer. *Cancer Res* **73**, 5880-5891, doi:10.1158/0008-5472.CAN-13-0821 (2013).
146 Scioli, M. G. *et al.* Adipose-Derived Stem Cells in Cancer Progression: New Perspectives and Opportunities. *Int J Mol Sci* **20**, doi:10.3390/ijms20133296 (2019).
147 Dirat, B. *et al.* Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res* **71**, 2455-2465, doi:10.1158/0008-5472.CAN-10-3323 (2011).
148 Laurent, V. *et al.* Periprostatic adipocytes act as a driving force for prostate cancer progression in obesity. *Nat Commun* **7**, 10230, doi:10.1038/ncomms10230 (2016).
149 Ribeiro, R. J. *et al.* Tumor cell-educated periprostatic adipose tissue acquires an aggressive cancer-promoting secretory profile. *Cell Physiol Biochem* **29**, 233-240, doi:10.1159/000337604 (2012).

150 Pavlides, S. *et al.* The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle* **8**, 3984-4001, doi:10.4161/cc.8.23.10238 (2009).
151 Migneco, G. *et al.* Glycolytic cancer associated fibroblasts promote breast cancer tumor growth, without a measurable increase in angiogenesis: evidence for stromal-epithelial metabolic coupling. *Cell Cycle* **9**, 2412-2422, doi:10.4161/cc.9.12.11989 (2010).
152 Gazi, E. *et al.* Direct evidence of lipid translocation between adipocytes and prostate cancer cells with imaging FTIR microspectroscopy. *J Lipid Res* **48**, 1846-1856, doi:10.1194/jlr.M700131-JLR200 (2007).
153 Nieman, K. M. *et al.* Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med* **17**, 1498-1503, doi:10.1038/nm.2492 (2011).
154 Gharpure, K. M. *et al.* FABP4 as a key determinant of metastatic potential of ovarian cancer. *Nat Commun* **9**, 2923, doi:10.1038/s41467-018-04987-y (2018).
155 Shafat, M. S. *et al.* Leukemic blasts program bone marrow adipocytes to generate a protumoral microenvironment. *Blood* **129**, 1320-1332, doi:10.1182/blood-2016-08-734798 (2017).
156 Palm, W. & Thompson, C. B. Nutrient acquisition strategies of mammalian cells. *Nature* **546**, 234-242, doi:10.1038/nature22379 (2017).
157 Beloribi-Djefaflia, S., Vasseur, S. & Guillaumond, F. Lipid metabolic reprogramming in cancer cells. *Oncogenesis* **5**, e189, doi:10.1038/oncsis.2015.49 (2016).
158 Currie, E., Schulze, A., Zechner, R., Walther, T. C. & Farese, R. V., Jr. Cellular fatty acid metabolism and cancer. *Cell Metab* **18**, 153-161, doi:10.1016/j.cmet.2013.05.017 (2013).
159 Mukherjee, A., Kenny, H. A. & Lengyel, E. Unsaturated Fatty Acids Maintain Cancer Cell Stemness. *Cell Stem Cell* **20**, 291-292, doi:10.1016/j.stem.2017.02.008 (2017).
160 Pandey, P. R. *et al.* Resveratrol suppresses growth of cancer stem-like cells by inhibiting fatty acid synthase. *Breast Cancer Res Treat* **130**, 387-398, doi:10.1007/s10549-010-1300-6 (2011).
161 Zaidi, N. *et al.* Lipogenesis and lipolysis: the pathways exploited by the cancer cells to acquire fatty acids. *Prog Lipid Res* **52**, 585-589, doi:10.1016/j.plipres.2013.08.005 (2013).
162 Hao, J. *et al.* Circulating Adipose Fatty Acid Binding Protein Is a New Link Underlying Obesity-Associated Breast/Mammary Tumor Development. *Cell Metab* **28**, 689-705 e685, doi:10.1016/j.cmet.2018.07.006 (2018).
163 Pascual, G. *et al.* Targeting metastasis-initiating cells through the fatty acid receptor CD36. *Nature* **541**, 41-45, doi:10.1038/nature20791 (2017).
164 Khandekar, M. J., Cohen, P. & Spiegelman, B. M. Molecular mechanisms of cancer development in obesity. *Nat Rev Cancer* **11**, 886-895, doi:10.1038/nrc3174 (2011).
165 Rose, D. P., Komninou, D. & Stephenson, G. D. Obesity, adipocytokines, and insulin resistance in breast cancer. *Obes Rev* **5**, 153-165, doi:10.1111/j.1467-789X.2004.00142.x (2004).
166 Kasiappan, R. *et al.* Vitamin D suppresses leptin stimulation of cancer growth through microRNA. *Cancer Res* **74**, 6194-6204, doi:10.1158/0008-5472.CAN-14-1702 (2014).
167 Zheng, Q. *et al.* Leptin receptor maintains cancer stem-like properties in triple negative breast cancer cells. *Endocr Relat Cancer* **20**, 797-808, doi:10.1530/ERC-13-0329 (2013).
168 Kato, S. *et al.* Leptin stimulates migration and invasion and maintains cancer stem-like properties in ovarian cancer cells: an explanation for poor outcomes in obese women. *Oncotarget* **6**, 21100-21119, doi:10.18632/oncotarget.4228 (2015).
169 Holland, W. L. *et al.* Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. *Nat Med* **17**, 55-63, doi:10.1038/nm.2277 (2011).
170 Grossmann, M. E. *et al.* Obesity and breast cancer: status of leptin and adiponectin in pathological processes. *Cancer Metastasis Rev* **29**, 641-653, doi:10.1007/s10555-010-9252-1 (2010).

171 Renehan, A. G., Zwahlen, M. & Egger, M. Adiposity and cancer risk: new mechanistic insights from epidemiology. *Nat Rev Cancer* **15**, 484-498, doi:10.1038/nrc3967 (2015).
172 Dalamaga, M., Diakopoulos, K. N. & Mantzoros, C. S. The role of adiponectin in cancer: a review of current evidence. *Endocr Rev* **33**, 547-594, doi:10.1210/er.2011-1015 (2012).
173 Park, J., Euhus, D. M. & Scherer, P. E. Paracrine and endocrine effects of adipose tissue on cancer development and progression. *Endocr Rev* **32**, 550-570, doi:10.1210/er.2010-0030 (2011).
174 Hakimi, A. A. *et al.* An epidemiologic and genomic investigation into the obesity paradox in renal cell carcinoma. *J Natl Cancer Inst* **105**, 1862-1870, doi:10.1093/jnci/djt310 (2013).
175 Ito, R. *et al.* The impact of obesity and adiponectin signaling in patients with renal cell carcinoma: A potential mechanism for the "obesity paradox". *PLoS One* **12**, e0171615, doi:10.1371/journal.pone.0171615 (2017).
176 Mano, R. *et al.* Association between visceral and subcutaneous adiposity and clinicopathological outcomes in non-metastatic clear cell renal cell carcinoma. *Can Urol Assoc J* **8**, E675-680, doi:10.5489/cuaj.1979 (2014).
177 Choi, Y. *et al.* Body mass index and survival in patients with renal cell carcinoma: a clinical-based cohort and meta-analysis. *Int J Cancer* **132**, 625-634, doi:10.1002/ijc.27639 (2013).
178 Albiges, L. *et al.* Body Mass Index and Metastatic Renal Cell Carcinoma: Clinical and Biological Correlations. *J Clin Oncol* **34**, 3655-3663, doi:10.1200/JCO.2016.66.7311 (2016).
179 Hale, M. *et al.* Obesity triggers enhanced MDSC accumulation in murine renal tumors via elevated local production of CCL2. *PLoS One* **10**, e0118784, doi:10.1371/journal.pone.0118784 (2015).
180 James, B. R. *et al.* Diet-induced obesity alters dendritic cell function in the presence and absence of tumor growth. *J Immunol* **189**, 1311-1321, doi:10.4049/jimmunol.1100587 (2012).
181 Zi, X. *et al.* Effect of perineoplasm perinephric adipose tissues on migration of clear cell renal cell carcinoma cells: a potential role of WNT signaling. *Oncotarget* **7**, 53277-53288, doi:10.18632/oncotarget.10467 (2016).
182 Campo-Verde-Arbocco, F. *et al.* Human renal adipose tissue induces the invasion and progression of renal cell carcinoma. *Oncotarget* **8**, 94223-94234, doi:10.18632/oncotarget.21666 (2017).
183 Egeblad, M. & Werb, Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* **2**, 161-174, doi:10.1038/nrc745 (2002).
184 Endo, H. *et al.* Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis. *Gut* **60**, 1363-1371, doi:10.1136/gut.2010.235754 (2011).
185 Brandon, E. L. *et al.* Obesity promotes melanoma tumor growth: role of leptin. *Cancer Biol Ther* **8**, 1871-1879, doi:10.4161/cbt.8.19.9650 (2009).
186 Giordano, C. *et al.* Leptin increases HER2 protein levels through a STAT3-mediated up-regulation of Hsp90 in breast cancer cells. *Mol Oncol* **7**, 379-391, doi:10.1016/j.molonc.2012.11.002 (2013).
187 Gonzalez-Perez, R. R., Lanier, V. & Newman, G. Leptin's Pro-Angiogenic Signature in Breast Cancer. *Cancers (Basel)* **5**, 1140-1162, doi:10.3390/cancers5031140 (2013).
188 Yang, W. H. *et al.* Leptin promotes VEGF-C production and induces lymphangiogenesis by suppressing miR-27b in human chondrosarcoma cells. *Sci Rep* **6**, 28647, doi:10.1038/srep28647 (2016).
189 Wang, Y. *et al.* Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. *Cancer Res* **66**, 11462-11470, doi:10.1158/0008-5472.CAN-06-1969 (2006).
190 Otani, K. *et al.* Adiponectin suppresses tumorigenesis in Apc(Min)(/+) mice. *Cancer Lett* **288**, 177-182, doi:10.1016/j.canlet.2009.06.037 (2010).

191 Sun, Y. & Lodish, H. F. Adiponectin deficiency promotes tumor growth in mice by reducing macrophage infiltration. *PLoS One* **5**, e11987, doi:10.1371/journal.pone.0011987 (2010).
192 He, J. Y. *et al.* Adipocyte-derived IL-6 and leptin promote breast Cancer metastasis via upregulation of Lysyl Hydroxylase-2 expression. *Cell Commun Signal* **16**, 100, doi:10.1186/s12964-018-0309-z (2018).
193 Heikkila, K., Ebrahim, S. & Lawlor, D. A. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. *Eur J Cancer* **44**, 937-945, doi:10.1016/j.ejca.2008.02.047 (2008).
194 Gyamfi, J., Lee, Y. H., Eom, M. & Choi, J. Interleukin-6/STAT3 signalling regulates adipocyte induced epithelial-mesenchymal transition in breast cancer cells. *Sci Rep* **8**, 8859, doi:10.1038/s41598-018-27184-9 (2018).
195 Chen, G. L. *et al.* High fat diet increases melanoma cell growth in the bone marrow by inducing osteopontin and interleukin 6. *Oncotarget* **7**, 26653-26669, doi:10.18632/oncotarget.8474 (2016).
196 Donohoe, C. L., Lysaght, J., O'Sullivan, J. & Reynolds, J. V. Emerging Concepts Linking Obesity with the Hallmarks of Cancer. *Trends Endocrinol Metab* **28**, 46-62, doi:10.1016/j.tem.2016.08.004 (2017).
197 Bluher, M. Obesity: global epidemiology and pathogenesis. *Nat Rev Endocrinol* **15**, 288-298, doi:10.1038/s41574-019-0176-8 (2019).
198 Johansson, M. *et al.* The influence of obesity-related factors in the etiology of renal cell carcinoma-A mendelian randomization study. *PLoS Med* **16**, e1002724, doi:10.1371/journal.pmed.1002724 (2019).
199 Haggstrom, C. *et al.* Metabolic factors associated with risk of renal cell carcinoma. *PLoS One* **8**, e57475, doi:10.1371/journal.pone.0057475 (2013).
200 Wang, F. & Xu, Y. Body mass index and risk of renal cell cancer: a dose-response meta-analysis of published cohort studies. *Int J Cancer* **135**, 1673-1686, doi:10.1002/ijc.28813 (2014).
201 Bagheri, M., Speakman, J. R., Shemirani, F. & Djafarian, K. Renal cell carcinoma survival and body mass index: a dose-response meta-analysis reveals another potential paradox within a paradox. *Int J Obes (Lond)* **40**, 1817-1822, doi:10.1038/ijo.2016.171 (2016).
202 Sharma, S. V., Haber, D. A. & Settleman, J. Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. *Nat Rev Cancer* **10**, 241-253, doi:10.1038/nrc2820 (2010).
203 Brodaczewska, K. K., Szczylik, C., Fiedorowicz, M., Porta, C. & Czarnecka, A. M. Choosing the right cell line for renal cell cancer research. *Mol Cancer* **15**, 83, doi:10.1186/s12943-016-0565-8 (2016).
204 Rodrigues, A. R. *et al.* Peripherally administered melanocortins induce mice fat browning and prevent obesity. *Int J Obes (Lond)* **43**, 1058-1069, doi:10.1038/s41366-018-0155-5 (2019).
205 Svensson, P. A. *et al.* Characterization of brown adipose tissue in the human perirenal depot. *Obesity (Silver Spring)* **22**, 1830-1837, doi:10.1002/oby.20765 (2014).
206 Nagano, G. *et al.* Activation of classical brown adipocytes in the adult human perirenal depot is highly correlated with PRDM16-EHMT1 complex expression. *PLoS One* **10**, e0122584, doi:10.1371/journal.pone.0122584 (2015).
207 Wu, N. N. *et al.* Brown adipogenic potential of brown adipocytes and peri-renal adipocytes from human embryo. *Sci Rep* **6**, 39193, doi:10.1038/srep39193 (2016).
208 Jespersen, N. Z. *et al.* Heterogeneity in the perirenal region of humans suggests presence of dormant brown adipose tissue that contains brown fat precursor cells. *Mol Metab* **24**, 30-43, doi:10.1016/j.molmet.2019.03.005 (2019).
209 Szarvas, T., vom Dorp, F., Ergun, S. & Rubben, H. Matrix metalloproteinases and their clinical relevance in urinary bladder cancer. *Nat Rev Urol* **8**, 241-254, doi:10.1038/nrurol.2011.44 (2011).

210 De Palma, M., Biziato, D. & Petrova, T. V. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer* **17**, 457-474, doi:10.1038/nrc.2017.51 (2017).
211 Ribeiro, R. *et al.* Human periprostatic adipose tissue promotes prostate cancer aggressiveness in vitro. *J Exp Clin Cancer Res* **31**, 32, doi:10.1186/1756-9966-31-32 (2012).
212 Wu, Q. *et al.* Cancer-associated adipocytes: key players in breast cancer progression. *J Hematol Oncol* **12**, 95, doi:10.1186/s13045-019-0778-6 (2019).
213 Balaban, S. *et al.* Adipocyte lipolysis links obesity to breast cancer growth: adipocyte-derived fatty acids drive breast cancer cell proliferation and migration. *Cancer Metab* **5**, 1, doi:10.1186/s40170-016-0163-7 (2017).
214 Roos, W. P., Thomas, A. D. & Kaina, B. DNA damage and the balance between survival and death in cancer biology. *Nat Rev Cancer* **16**, 20-33, doi:10.1038/nrc.2015.2 (2016).
215 Helleday, T., Petermann, E., Lundin, C., Hodgson, B. & Sharma, R. A. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* **8**, 193-204, doi:10.1038/nrc2342 (2008).
216 Hoeijmakers, J. H. Genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366-374, doi:10.1038/35077232 (2001).
217 Takahara, K. *et al.* Adipose-derived stromal cells inhibit prostate cancer cell proliferation inducing apoptosis. *Biochem Biophys Res Commun* **446**, 1102-1107, doi:10.1016/j.bbrc.2014.03.080 (2014).
218 Kelesidis, I., Kelesidis, T. & Mantzoros, C. S. Adiponectin and cancer: a systematic review. *Br J Cancer* **94**, 1221-1225, doi:10.1038/sj.bjc.6603051 (2006).
219 Ogrodnik, M. *et al.* Obesity-Induced Cellular Senescence Drives Anxiety and Impairs Neurogenesis. *Cell Metab* **29**, 1233, doi:10.1016/j.cmet.2019.01.013 (2019).
220 Saleh, T., Tyutyunyk-Massey, L. & Gewirtz, D. A. Tumor Cell Escape from Therapy-Induced Senescence as a Model of Disease Recurrence after Dormancy. *Cancer Res* **79**, 1044-1046, doi:10.1158/0008-5472.CAN-18-3437 (2019).
221 Cao, Y. Adipocyte and lipid metabolism in cancer drug resistance. *J Clin Invest* **129**, 3006-3017, doi:10.1172/JCI127201 (2019).